

**RIBOSOMAL PROTEIN MUTANTS AND THEIR EFFECTS ON PLANT GROWTH AND  
DEVELOPMENT**

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By

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## ABSTRACT

Ribosomes, large enzymatic complexes containing an RNA catalytic core, drive protein synthesis in all living organisms. 80S cytoplasmic eukaryotic ribosomes are comprised of four rRNAs and approximately 80 ribosomal proteins (r-proteins). R-proteins are encoded by gene families with large families (average of twelve members) predominating in mammals and smaller families (two to seven members) in plants. Increased ribosome heterogeneity is possible in plant ribosomes due to multiple transcriptionally active members in each family, whereas, in mammalian r-protein gene families, only one member is typically active. Multiple functional paralogs provide for greater plasticity in response to environmental/developmental cues, as well as, increasing the possibility of individual paralogs procuring or retaining extraribosomal functions. This research investigated the effects of r-protein mutations on plant growth and development. Through RNA interference (RNAi) mediated knockdown (KD) of type I (cytoplasmic: *RPS15aA/D* and *F*) and type II (non-cytosolic: *RPS15aB* and *E*) *RPS15a* family members I was able to confirm the delineation between the two types. Subcellular localization of the type I isoforms was nuclear/nucleolar while localization of type II isoforms was non-mitochondrial and probably cytosolic. Illumina sequencing of two r-protein mutant transcriptomes, *pfl1* (*rps18a*) and *pfl2* (*rps13a*), identified a novel set of up and down regulated genes, previously unknown or linked to r-protein mutants. The 20 genes identified were classified into four groups (1) plant defense, (2) transposable elements, (3) nitrogen metabolism and (4) genes with unknown function. Illumina miRNOME analysis revealed no changes in the miRNA profile of *pfl1* and *pfl2* plants. These data do not support the previously proposed theory that a disruption in ribosome biogenesis (by decreased r-protein synthesis) disrupts miRNA-mediated degradation of a range of auxin response genes. Finally, a novel double r-protein mutant, *rps18a:HF/RPL18B*, presented a late flowering/thickened bolt phenotype not seen in a *rps13a:HF/RPL18B* mutant, suggesting that RPS18A has an extraribosomal role in plant growth and development in Arabidopsis.

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## **DEDICATION**

For their constant love, support and encouragement throughout this endeavor I dedicate this thesis to my wife Rebeccah Molnar and parents Gord and Cheryl Stewart.

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## LIST OF ABBREVIATIONS

### Abbreviation

ABA	abscisic acid
ABRC	Arabidopsis Biological Resource Center
A site	aminoacyl site
BAP	6-Benzylaminopurine/cytokinin
bp	base pair
cDNA	complementary DNA
CDS	coding sequence
CLSM	confocal laser scanning microscope
DAPI	4',6-diamidino-2-phenylindole
DFC	dense fibrillar component
E site	exit site
ETS	external transcribed sequence
FC	fibrillar center
GA	gibberellic acid
GFP	green fluorescent protein
hnRNP	heteronuclear ribonucleoparticle
IAA	indole acetic acid
ITS	internal transcribed sequence
Kan	kanamycin
KD	knockdown
KO	knockout
LB	lysogeny broth
LC-MS/MS	liquid chromatography tandem mass spectroscopy
LSU	large subunit
miRNA	micro RNA
mRNA	messenger RNA
MS	Murashige and Skoog
NES	nuclear export sequence
NLS	nuclear localization signal



NMR	nuclear magnetic resonance
NoLS	nucleolar localization signal
NOR	nucleolar organizer region
NPC	nuclear pore complex
ORF	open reading frame
pI	isoelectric point
pfl	pointed first leaf
P site	peptidyl site
qRT-PCR	real-time quantitative RT- PCR
RACK	receptor of activated C-kinase
rDNA	ribosomal DNA
RFP	red fluorescent protein
RNAi	RNA interference
RNP	ribonucleoprotein
RNA Pol I	RNA polymerase I
r-proteins	ribosomal proteins
rRNA	ribosomal RNA
SAIL	Syngenta Arabidopsis Insertion Library
SALK	Institute for Biological Studies
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	small interfering RNA
snRNP	small nuclear RNP
snoRNA	small nucleolar RNA
snoRNP	small nucleolar RNP
SSU	small subunit
tRNA	transfer RNA
UAS	upstream activation sequence
UTR	untranslated region
WT	wild type

## CHAPTER 1. LITERATURE REVIEW

### 1.1 Introduction

Ribosome biology has focused primarily on prokaryotic (e.g. *E. coli*) and several select eukaryotic systems (human and *Saccharomyces cerevisiae*), however, these organisms do not have the ribosome complexity of plants. With three distinct ribosome populations (80S cytosolic and 70S mitochondrial and plastid) and multiple expressed members from each ribosomal protein (r-protein) gene family, the possible level of ribosome heterogeneity found in plant ribosomes is unmatched.

With the discovery by the Steitz group (2000) that the peptidyl transferase activity of the large subunit is the result of ribosomal RNA (rRNA) catalytic activity, the ribosome is now referred to as a 'ribozyme' (Kruger et al., 1982; Nissen et al., 2000; Hansen et al., 2001). This discovery supports the theory that early ribosomes were comprised entirely of RNA to which at a later date, proteins with specialized functions were recruited (Nissen et al., 2000). Although not directly required for enzymatic activity in the ribosome, r-proteins are still essential for numerous roles both integral to the ribosome (mRNA and tRNA decoding, mRNA binding, scaffolding and peptidyl transferase cofactors) (Brodersen and Nissen, 2005) and away from the ribosome (translational regulation, DNA repair, cell cycle regulation and transcription factor activity) (Yates et al., 1980; Kim et al., 1995; Zhang and Lu, 2009; Wang et al., 2011).

I have used r-protein gene manipulation to try and elucidate the role of r-proteins in normal plant growth and development, as well as trying to identify the importance of the individual members of the unique *RPS15a* gene family in Arabidopsis. To determine cellular requirements for individual family members and type I and II families of the small subunit *RPS15a* gene family in Arabidopsis, RNAi mediated knockdowns (KDs) of each member and family type were generated. Subcellular localization of all proteins encoded by the *RPS15a* family was also determined. To monitor the effect on plant growth and development of concomitant reductions of *RPS18A* or *RPS13A* with an overproduction of *RPL18B*, the *rps18a/RPL18B* and *rps13a/RPL18B* double mutants were generated. Subcellular localization of proteins encoded by the *RPS18*, *RPS13* and *RPL18* gene families were determined. Illumina sequencing was used to determine the effect on both the seedling transcriptome and miRNOME of *rps18a* and *rps13a* mutants. To determine if there

is a similar subset of genes up or down regulated in both r-protein and auxin mutants, a number of such identified genes in the r-protein mutants (*rps18a* and *rps13a*) were investigated by qRT-PCR in *tir1*, *afb1*, *afb2* and *afb3* (auxin mutants).

## **1.2. Ribosome Structure and Function**

Ribosomes are large, enzymatic, macromolecular complexes, comprised of two subunits of unequal sizes, which are essential for protein synthesis in all living organisms. In eukaryotes, the 80S ribosome contains a 60S (26S, 5S and 5.8S ribosomal RNA (rRNA) and ~45 r-proteins) large subunit (LSU) and 40S (18S rRNA and ~ 33 r-proteins) small subunit (SSU), while in prokaryotes the smaller 70S ribosome contains a 50S (23S and 5S rRNA and 31 r-proteins) LSU and 30S (16S rRNA and 21 r-proteins) SSU (Barakat et al., 2001; McIntosh and Bonham-Smith, 2006). As the sole protein-synthesizing complex in the cell, the mechanism of nascent protein production has received a great deal of attention. These massive ribonucleoprotein particles contain three binding sites for transfer RNAs (tRNA): the A (aminoacyl) site that accepts aminoacylated tRNA, the P (peptidyl) site that holds tRNA with the growing polypeptide chain and catalyzes peptide bond formation between amino acids and the E (exit) site that is responsible for holding the deacylated tRNA before it exits the ribosome (Ramakrishnan, 2002; Schmeing and Ramakrishnan, 2009).

Both the LSU and SSU exist freely in the cell until binding to a messenger RNA (mRNA) transcript occurs, at which time a complex scaffold of assembly proteins coalesce and the ribosome becomes translationally competent. Initially the SSU binds the transcript, as well as, the anticodon stem loop of the aminoacylated tRNA. The small subunit also possesses some proofreading ability as it monitors complementary base pairing between the mRNA codon and aminoacylated tRNA anticodon during synthesis. The LSU 26S/23S rRNA possesses the peptidyl transferase activity of the ribosome and is responsible for catalyzing peptide bond formation between amino acids of nascent polypeptides (Schmeing and Ramakrishnan, 2009).

### **1.3. Ribosome Assembly**

#### **1.3.1. Ribosome Components**

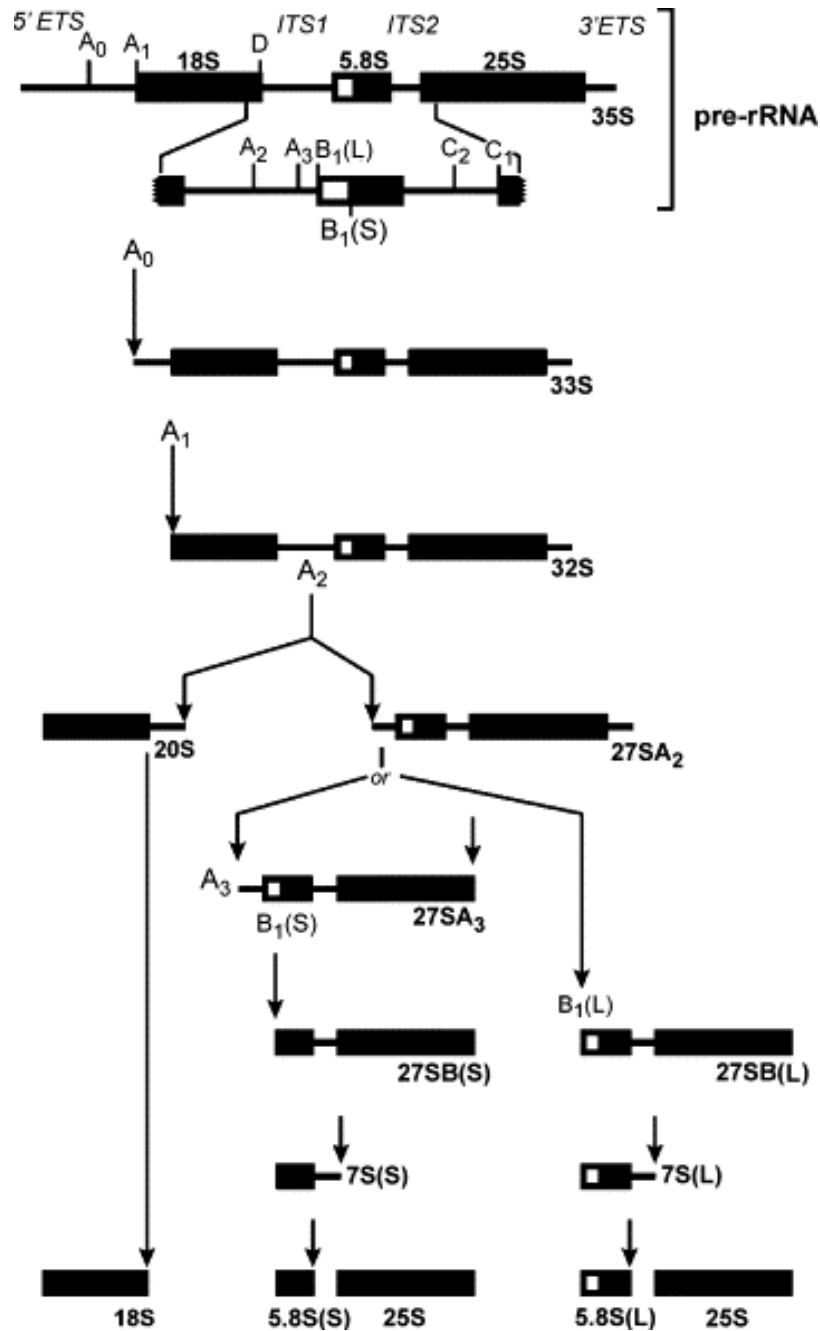
Cytoplasmic ribosome subunit assembly is a complex process occurring in the nucleolus and requiring the coordinated efforts of more than 180 assembly factors and 100 small nucleolar ribonucleoprotein particles (snoRNPs: required to catalyze post-transcriptional modifications of pre-rRNA) (Decatur and Fournier, 2003). rRNA must be synthesized, folded and modified (methylated or pseudouridylated), processed by exo- and endonucleases and bound to r-proteins (Henras et al., 2008). In general, three of the four rRNA genes (26S, 18S and 5.8S rRNA) are found as a single transcriptional unit in nucleolar organizer regions (NORs) of the genome and are transcribed by RNA polymerase I (RNA Pol I: performs up to 80% of transcription in rapidly growing cells) (Schafer et al., 2003; Staley and Woolford, 2009). These repeats consist of a 5' external transcribed sequence (ETS), 18S rDNA, internal transcribed sequence 1 (ITS), 5.8S rDNA, ITS2, 26S rDNA and 3' ETS, all of which is initially transcribed into a 35S pre-rRNA transcript in the nucleolus. 5S rRNA genes, located near the centromeres of chromosomes three, four and five in Arabidopsis (Kaul et al., 2000), are transcribed as pre-5S rRNA by RNA Pol III in the nucleus and are then transported to the nucleolus (McIntosh and Bonham-Smith, 2006; Staley and Woolford, 2009). In Arabidopsis, r-protein genes are distributed across all five chromosomes (Barakat et al., 2001). Transcription occurs in the nucleus by RNA Pol II, with the resulting transcripts transported to the cytosol for translation and the majority of synthesized r-proteins transported back to the nucleolus for pre-ribosomal subunit assembly (Schafer et al., 2003; McIntosh and Bonham-Smith, 2006; Staley and Woolford, 2009).

#### **1.3.2. Ribosomal Subunit Biogenesis**

The pre-35S rRNA and pre-5S rRNA transcripts undergo a great deal of processing during subunit biogenesis that requires a complex array of proteins including snoRNPs, exo- and endonucleases (essential for pre-rRNA processing), pseudouridine synthases and methyltransferases (required for covalent modifications) and helicases and chaperones (for proper RNA folding) (Schafer et al., 2003). Subunit assembly and pre-rRNA processing occur co-transcriptionally with certain assembly factors interacting with both pre-rRNA

and rDNA (Staley and Woolford, 2009). The production of subcomplexes aids in the assembly of ribosomal RNPs and in yeast, five such subcomplexes have been identified; tUTP/UTP-A, UTP-B, UTP-C, Mpp10 and the U3 complex (Schafer et al., 2003). A number of non-ribosomal proteins associated with the U3 complex; Nop56p, Nop58p, Sof1p, Rrp9p, Dhrip, Imp3p, Imp4p and Mpp10p, and factors specific for 18S rRNA processing, have been identified in the 90S pre-ribosome and are required for SSU biogenesis, with LSU factors binding later (Grandi et al., 2002; Schafer et al., 2003). In yeast, the 35S pre-rRNA in the 90S preribosome undergoes a series of enzymatic cleavages to produce three of the four rRNAs found in an active ribosome (Figure 1.1); twice at sites A<sub>0</sub> and A<sub>1</sub> to remove the 5' ETS and yield a 32S pre-rRNA and once within ITS1 at site A<sub>2</sub> to generate 20S (precursor to 18S rRNA) and 27S (precursor to 5.8S and 26S rRNA) pre-RNA (Granneman and Baserga, 2004). Following transport of the 20S pre-rRNA in association with SSU r-proteins as a 43S pre-40S particle to the cytoplasm, a final cleavage at site D releases the ITS1 tail and a fully functional 40S SSU is the result. In most cases, the 27S pre-rRNA is first cleaved by RNase MRP at A<sub>3</sub>, although not absolutely required, then B<sub>1</sub>(S) or B<sub>1</sub>(L) sites producing 27SA<sub>3</sub> precursor followed by cleavage within the ITS2 at C<sub>1</sub> and C<sub>2</sub> sites to produce mature 5.8S(S) and 26S rRNA or 5.8S(L) and 26S rRNA. These two rRNAs are assembled with 5S rRNA into a 66S pre-60S particle and subsequently transported to the cytoplasm for final maturation into a functional 60S ribosomal subunit (Brown and Shaw, 1998; Schafer et al., 2003; Granneman and Baserga, 2004; Rouquette et al., 2005).

Processing of the 35S pre-rRNA into 20S and 27S pre-rRNA by cleavage at the A<sub>0</sub>, A<sub>1</sub> and A<sub>2</sub> sites requires the SSU processome (terminal knob: a large snoRNP) that assembles co-transcriptionally at the 5' end of 35S pre-rRNA (Schafer et al., 2003; Bernstein et al., 2004). The SSU processome is comprised of U3 small nucleolar RNA (snoRNA), U3 binding proteins and 17 additional proteins (Utp1-17) with unknown functions (Kressler et al., 1999; Dragon et al., 2002; Bernstein et al., 2004). Two different classes of snoRNPs are required for normal ribosome biogenesis: (1) H/ACA box snoRNAs direct site-specific pseudouridylation of rRNA, (2) C/D box snoRNAs target specific rRNA nucleotides for 2'-O-ribose methylation and for mitochondrial ribosomes a third class is required (3) RNase mitochondrial RNA processing snoRNPs (Bernstein et al., 2004)



**Figure 1.1. *S. cerevisiae* pre-rRNA processing.** 35S pre-rRNA (containing 18S, 5.8S and 25S rRNA precursors) is transcribed as a single unit by RNA Pol I. It is cleaved at sites A<sub>0</sub>, A<sub>1</sub> and A<sub>2</sub> to yield the 20S (18S rRNA precursor) and 27S (5.8S and 25S rRNA precursors) pre-rRNAs. The 20S pre-rRNA is exported to the cytoplasm and matures to 18S rRNA following cleavage at site D. In the nucleus, 27S pre-rRNA undergoes final processing at site A<sub>3</sub> (or site B) within ITS2 to give mature 5.8S and 26S rRNAs (Modified from Granneman and Baserga, 2004).

### **1.3.3. Ribosomal Subunit Export**

During transport from the nucleolus to the nucleus, preribosomal subunits undergo the final steps in maturation to become functional ribosomal subunits. Prior to release into the cytoplasm, the preribosomal subunits must be of a size that facilitates passage through nuclear pores. In yeast, this is achieved by the release of assembly factors and conformational changes of the pre-RNPs (Nissan et al., 2002; Schafer et al., 2006; Kemmler et al., 2009), with passage mediated by a variety of factors including Crm1/Xpo1 receptors that recognize the transport factors; Nmd3 (pre-60S particle) and Ltv1, DIM2 and RIO2 (pre-40S particles) (Seiser et al., 2006; Zemp et al., 2009) containing nuclear export sequences (NESS), Ran-GTP (Ho and Johnson, 1999; Ho et al., 2000; Gadad et al., 2001; Bradatsch et al., 2007; Yao et al., 2007; Hung et al., 2008), the Nup82 complex comprised of Nup82p, Nup159p, Nsp1p, Nup116p and Gle2p (Hurt et al., 1999; Moy and Silver, 1999; Gleizes et al., 2001; Moy and Silver, 2002) and the shuttling factors Rrp12 (HEAT repeat containing protein) and Mex67-Mtr2 (mRNA export factor) (Oeffinger et al., 2004; Yao et al., 2008). Whereas most export factors dissociate from the pre-60S particle in the nucleus/nucleolus, a subset including Nmd3, Rlp24, Tif6, Nog1, Arx1 and Alb1, are required for final 60S maturation in the cytoplasm (Saveanu et al., 2001; Senger et al., 2001; Hedges et al., 2005; Hung and Johnson, 2006; Lebreton et al., 2006; Pertschy et al., 2007). In the final step to becoming translationally competent LSUs, pre-60S particles associate with the cofactors Kre35, Efl1, Sdo1, Rei1 and Drg1 (Senger et al., 2001; Saveanu et al., 2003; Hedges et al., 2005; Hung and Johnson, 2006; Lebreton et al., 2006; Menne et al., 2007; Pertschy et al., 2007; Zemp and Kutay, 2007) and the few remaining r-proteins (RPL10, RPL24 and the acidic r-protein Rpp0) (Zinker and Warner, 1976; Kruiswijk et al., 1978; Saveanu et al., 2003; Kressler et al., 2008).

### **1.4. The Nucleolus**

Individual nucleoli form during interphase around active NORs and in plants, generally fuse together to form a single nucleolus (Shaw and Jordan, 1995). Lacking a membrane, this subnuclear, dynamic structure breaks down at the end of G2 and upon reassembly can change shape, size and position within the nucleus (Shaw and Brown, 2012). The nucleolus is the site of 26S, 5.8S and 18S rDNA transcription, rRNA maturation

and ribosomal subunit assembly (Shaw and Brown, 2012) and in association with cajal bodies, are also essential for the maturation, assembly and export of the signal recognition particle, telomerase RNP, the processing of precursor tRNA and U6 small nuclear RNAs (snRNAs), regulating the cell cycle, stress responses and telomerase activity (Pederson, 1998; Tsai and McKay, 2002; Rubbi and Milner, 2003; Olson, 2004; Raska et al., 2006; Boisvert et al., 2007; Boulon et al., 2010).

A nucleolus is a tripartite structure in amniotes, comprised of a fibrillar center (FC), a dense fibrillar component (DFC) and a granular component (GC), while anamniote nucleoli lack FCs (Shaw et al., 1995; Raska, 2003; Raska et al., 2006). Containing little RNA, the FC does contain a high concentration of rDNA and is encircled by a DFC that contains high concentrations of RNA and is the site of the first steps of pre-rRNA processing (Carmo-Fonseca et al., 2000; Huang, 2002; Olson et al., 2002). The FC and DFC are located within a GC, the latter required for late stage pre-rRNA processing (Shaw et al., 2002; Raska, 2003).

Currently, 4500 proteins have been identified in animal nucleoli including some known to be involved in ribosome biogenesis (fibrillarin, nucleolin and B23) as well as splicing factors, spliceosomal proteins and translation factors (Andersen et al., 2002; Scherl et al., 2002; Ahmad et al., 2009). Characterization of proteins associated with the plant nucleolus is not as advanced with only 217 proteins identified to date from purified *Arabidopsis* nucleoli (Pendle et al., 2005). As in animal nucleoli, these include ribosomal and nucleolar proteins and splicing and translation factors, but also some plant specific proteins and proteins with unknown function were identified (Pendle et al., 2005). The RNA composition of the animal nucleolus includes snoRNAs, snRNAs, tRNAs, 7SL RNA (signal recognition pathway) and telomerase RNA (Shaw and Brown, 2012). A similar RNA composition has been identified in plant nucleoli along with numerous mRNAs (Kim et al., 2010) and small-regulatory RNAs, hinting at possible novel nucleolar function in gene regulation in plants (Shaw and Brown, 2012).

The nucleolus (or associated processing (P)-bodies) is also both the site of synthesis of heterochromatic small interfering RNAs (siRNAs) and the site of precursor miRNA localization (Pontes and Pikaard, 2008), further suggesting that it is involved in posttranscriptional regulation of gene expression (Shaw and Brown, 2012).



## 1.5. Ribosomal Proteins

Until recently, r-proteins were thought of as purely housekeeping proteins, required for basic architectural roles in the ribosome. However, with the identification of an upregulation of r-protein expression during cold treatment (Saez-Vasquez et al., 2000; Kim et al., 2004), UV treatment (Casati and Walbot, 2003) and over a variety of developmental stages (Taylor et al., 1992; Vanlijsebettens et al., 1994), it has now been recognized that r-proteins play a more substantial role in plant growth and development. Since this paradigm shift, r-proteins RPS6, RPS11, RPL2, RPL8, RPL23, RPL19, RPL23aA and RPL40 have all been identified as absolutely required for normal plant growth (Tzafrir et al., 2003; Tzafrir et al., 2004; Degenhardt and Bonham-Smith, 2008; Meinke et al., 2008). In mammals, a growing number of r-proteins, including RPL5, RPL7, RPL11, RPL23, RPL26, RPL29, RPL30, RPL37, RPS3, RPS6, RPS7, RPS9 and RPS27 have been shown to indirectly influence the cell cycle through interactions in the p53-MDM2 pathway, where changes in any of these r-proteins can lead to tumorigenesis (Chen et al., 2007; Horn and Vousden, 2008; Fumagalli et al., 2009; Yadavilli et al., 2009; Zhang and Lu, 2009; Zhu et al., 2009; Daftuar et al., 2010; Xiong et al., 2011).

In Arabidopsis, r-protein genes are present on all five chromosomes and are transcribed in the nucleus by RNA Pol II. R-protein mRNAs are subsequently transported to the cytosol for translation and the resulting proteins are transported back through the nucleus to the nucleolus for pre-ribosomal subunit assembly. The resulting pre-ribosomal subunits are then transported to the cytosol via nuclear pores during which time, final rRNA processing occurs such that assembly on transcripts, into functional ribosomes, is now possible.

R-proteins can account for one-third to one-half the weight of a ribosome. Individually, they are small, basic (due to high lysine and arginine and low aspartate and glutamate content;  $pI > 8$ ) (Barakat et al., 2001) RNA binding proteins ranging in size from 4 to 30 kDa in *E. coli* (Arnold and Reilly, 1999) and 3.4 to 47 kDa in rat (Wool et al., 1995) and Arabidopsis (Barakat et al., 2001; Chang et al., 2005). The r-protein to rRNA ratio increases from prokaryotes to eukaryotes with 55 r-proteins in *E. coli* (Kaltschm.E and Wittmann, 1970), 78 in *S. cerevisiae* (Mager et al., 1997; Planta and Mager, 1998), 80 in *M.*

*musculus* (Wool et al., 1995) and *H. sapiens* (Uechi et al., 2001) and 75-92 in plants (81 in *Arabidopsis*) (Bailey-Serres et al.; Barakat et al., 2001).

R-proteins are highly conserved among the three domains (Bacteria, Archaea and Eukarya). While eukaryotic cytosolic r-proteins have an archaeobacterial ancestry (Matheson et al., 1990; Wittmannliebold et al., 1990; Wool et al., 1995), plastid and mitochondrial r-proteins have a eubacterial ancestry (Graack and Wittmann-Liebold, 1998; Koc et al., 2000; Yamaguchi and Subramanian, 2000; Yamaguchi et al., 2000). Eukaryotic r-proteins can further be classified into three groups based on ancestral homologies to archaea and bacterial r-proteins. Group one contains orthologs found in all three domains, Group two orthologs are found in archaeobacteria and eukaryotes, and Group three contains solely eukaryotic orthologs (Wool et al., 1995; Barakat et al., 2001).

In an attempt to investigate the evolutionary distribution of r-proteins, Lecompte et al. (2002) studied the genomes of 66 completely sequenced organisms from the three domains of life. Confirming the structural and functional similarity between prokaryotic and eukaryotic ribosomes, 32 families were conserved in all three domains while 23 families were specific to Bacteria and 33 families were specific to Archaea and Eukarya. Only one family, not found in Eukarya, was specific to Archaea and no specific r-proteins were found between Bacteria and Archaea or Bacteria and Eukarya, underlying the early divergence of Bacteria from both Archaea and Eukarya (Lecompte et al., 2002). Four r-proteins (S1p, S21p, L25p and L30p), in a small group of Bacteria from free-living bacteria to intracellular pathogens, show a disparate distribution suggesting that these gene losses occurred independently in these species (Lecompte et al., 2002). The highest degree of r-protein conservation is seen in Eukarya, with most r-proteins represented in all analyzed genomes with the exception of *Encephalitozoon cuniculi* (amitochondriate Microsporidia: an obligate intracellular parasitic fungi thought to be an early eukaryotic lineage diverging prior to the incorporation of mitochondria) in which four r-proteins (S21e, L28e, L14e and L38e) are absent (Vossbrinck et al., 1987; Cavaliersmith, 1989). The absence of these four r-protein genes was attributed to the early divergence of *E. cuniculi* (Lecompte et al., 2002). In contrast to Bacteria and Eukarya, the Archaea genomes examined exhibited a low degree of conservation, with losses of up to ten r-protein genes in Crenarchaeota, suggesting that

the Archaea domain is demonstrating uncommon reductive evolution in a non-eukaryotic species (Lecompte et al., 2002).

### **1.5.1. Ribosomal Protein Functions**

Prior to determination of the structure of the bacterial 70S ribosome (atomic structures of both the 50S at 2.4Å and 30S at 3.3 and 3.0Å were determined in 2000 (Ban et al., 2000; Schlueder et al., 2000; Wimberly et al., 2000) and once the rRNA was shown to be enzymatic, it was postulated that r-proteins were primarily structural, ensuring proper rRNA folding and, therefore, proper rRNA function. Due to the tight interactions with rRNA, isolating and crystallizing individual r-proteins has been extremely difficult and when accomplished, has yielded only local r-protein-rRNA interactions (Brodersen and Nissen, 2005). However, crystallized structures for both bacterial LSU and SSU have confirmed the location of r-proteins on the surface of the ribosome with the rRNAs comprising the catalytic core (Ban et al., 2000; Schlueder et al., 2000; Wimberly et al., 2000). These crystallized structures have identified long peptide tails at the termini of r-proteins or as internal loop structures that appear to function as anchors to the rRNA core increasing the surface area for r-protein-rRNA interactions (Ban et al., 2000; Wimberly et al., 2000; Brodersen et al., 2002; Klein et al., 2004; Brodersen and Nissen, 2005).

Prior to crystallization of the 70S ribosome (Ban et al., 2000; Schlueder et al., 2000; Wimberly et al., 2000), mutational analysis of a number of prokaryotic r-proteins had identified their importance in a variety of ribosomal processes. RPS12 was shown to be required for tRNA decoding in the ribosomal A site (Funatsu and Wittmann, 1972) and *RPS4* and *RPS5* mutations conferred streptomycin resistance and ribosome translational inaccuracy (Deusser et al., 1970; Stoffler et al., 1971). RPS9 and RPS13 interact with the P site tRNA in the 30S subunit while RPL2 and RPL3 are required for optimal peptidyl transferase activity of the 50S subunit by stabilizing the 23S rRNA (Brodersen and Nissen, 2005). RPL4 and RPL22 form a narrow constriction in the polypeptide exit tunnel of the ribosome allowing for polypeptide sequence targeting or possibly monitoring the functionality of the ribosomal state (Brodersen and Nissen, 2005). Two additional r-proteins, RPL22 and RPL39e also line the polypeptide exit tunnel and may facilitate movement of the nascent polypeptide through the tunnel. At the tunnel exit area,

interactions between the signal recognition particle (SRP) and the new polypeptide are enhanced by RPL22 and RPL23a, which anchor the SRP and the trigger factor (Kramer et al., 2002; Gu et al., 2003), RPL24 and RPL29 (Brodersen and Nissen, 2005). In the SSU, RPS1, RPS7 and RPS11 are essential for mRNA binding, primarily tethering mRNA to the ribosome (Brodersen and Nissen, 2005).

Export of mRNA from transcriptional regions to ribosomes differs in prokaryotes and eukaryotes, as the latter requires the mRNA to be packaged as heteronuclear ribonucleoparticle (hnRNP) complexes prior to export from the nucleus. A hnRNP is comprised of numerous RNA-binding proteins that coat the mRNA and function in signaling pathways linking mRNA to translation initiation (Brodersen and Nissen, 2005). Perhaps the most important of these proteins is receptor of protein kinase C (RACK1), which is an r-protein located near to the mRNA at the E site of the small subunit (Link et al., 1999). RACK1 binds protein kinase C (activating translation), Src kinase, Scp160p (mRNA-binding protein) and integrin  $\beta$  (receptor) suggesting that it is involved in mRNA binding and translation initiation on the ribosome (Nilsson et al., 2004; Brodersen and Nissen, 2005). In prokaryotes, several small and large subunit r-proteins can, together, function as a binding site for GTP-containing translation factors. In conjunction with the sarcin-ricin loop and RPL11 RNA region (GTPase associated center), RPS4, RPL6, RPL14, RPL11 and the acidic stalk proteins RPL10 and RPL7/RPL12 form the GTP containing translation factor-binding site. For example, r-proteins comprising this site make direct contact with elongation factor EF-G and the aminoacyl tRNA-EF-Tu ternary complex (Valle et al., 2003), although how the GTPase activity of these binding factors is activated is still unclear (Brodersen and Nissen, 2005). Finally, r-proteins have roles in the nuclear export of 5S rRNA and both small and large subunits to the cytoplasm (Guddat et al., 1990; Ho et al., 2000; Gadal et al., 2001; Leger-Silvestre et al., 2004).

## **1.5.2. R-protein Gene Regulation**

### **1.5.2.1 R-Protein Gene Arrangement**

R-protein genes in prokaryotes are predominantly arranged as operons (multiple genes under the control of a single promoter) with most r-protein genes located within 20

operons (*E. coli*) (Mager, 1988). In plants (rice, maize and Arabidopsis), there is a tendency for smaller but more transcriptionally active r-protein gene families than found in mammals (rat and human), while yeast r-protein gene families, on average, contain only two members (Warner et al., 1985; Wu et al., 1995). In contrast, the r-protein genes in prokaryotes are predominantly present as single copies (Makarova et al., 2001).

In the yeast *S. cerevisiae*, 137 genes dispersed throughout the genome encode 78 r-proteins with 59 of the 78 encoded by duplicate functional genes (Warner et al., 1985; Planta and Mager, 1998; Jimenez et al., 2003). In Arabidopsis, 254 r-protein genes are arranged in gene families with two to seven members (average of three) that encode 81 r-proteins, suggesting that the expression of various isoforms from each family, of which only one copy of one isoform (except the acidic P r-proteins) is incorporated into any one ribosome, may be related to stress, developmental or environmental pressures (Barakat et al., 2001; Degenhardt and Bonham-Smith, 2008). In contrast to yeast and plants, with their small actively transcribed gene families, mammalian gene families are large, averaging 12 members each in rat, but with a single active member (Wool et al., 1995). The other members of each family are predominantly inactive pseudogenes such as in humans where there are upwards of 2000 processed r-protein pseudogenes in the genome (Harrison et al., 2002; Zhang et al., 2002). In plants, multiple expressed members of the same gene family for each r-protein may be required to accommodate the rapid growth associated with a variety of development stages. Therefore, having multiple independently transcribed family members for each r-protein may optimize ribosome biogenesis and thus protein synthesis during these times of rapid growth. Retaining multiple active r-protein gene family members in plants may also lead to ribosomal heterogeneity resulting in functional specialization of ribosomes for a myriad of developmental or environmental situations (Gilbert, 2011). Increased ribosome plasticity would improve a plant's ability to positively respond to adverse conditions and allow for a high degree of "fine tuning" in mRNA selection for translation.

#### **1.5.2.2. Prokaryotic R-Protein Gene Regulation**

In prokaryotes, r-protein genes are arranged in operons with up to half of all r-protein genes being located in three operons; the *S10*, *spc* and *alpha* operons (Lindahl and

Zengel, 1986; Mager, 1988; Coenye and Vandamme, 2005). These operon gene arrangements enable rapid responses to changing environmental/developmental conditions resulting in rapid modulation of r-protein production and thus, ribosome biogenesis and protein synthesis. Autogenous regulation of prokaryotic r-protein gene operons predominates. The translation of one of the proteins in the operon acts as a negative regulator of that operon either through transcriptional (binding to leader sequences) or translational (binding polycistronic mRNA) repression (Lindahl and Zengel, 1986). In *E. coli*, RPL1, RPS4 and RPS8 regulate the *L11*, *alpha* and *spc* operons, respectively (Yates et al., 1980), RPS7 regulates the *str* operon (Nomura et al., 1980) and RPL20 regulates the *IF3* operon (Raibaud et al., 2003). Crystallography of RPS8 (Merianos et al., 2004) and RPL1 (Nevskaya et al., 2005) and nuclear magnetic resonance (NMR) imaging of RPL20 (Raibaud et al., 2003) have provided insights into how prokaryotic r-proteins regulate their own translation. RPS8, RPL1 and RPL20 can bind to similar RNA structures in rRNA and mRNA, although of the two, each r-protein has a higher affinity for their rRNA to ensure efficient ribosome biogenesis (Lindahl and Zengel, 1986).

### **1.5.2.3 Eukaryotic R-Protein Gene Regulation**

#### **1.5.2.3.1 Yeast R-Protein Gene Regulation**

With the exception of a few cases, RPL2 and RPL30, of autonomous regulation of yeast r-protein genes, (Presutti et al., 1995; Mao et al., 1999; Vilardell et al., 2000) transcriptional regulation is the norm. Two upstream activation sequences (UAS), the RPG and HOMOL1 boxes (Mager, 1988), in the 5' UTR and the repressor-activator protein 1 (Rap1), a DNA binding protein capable of both activating and repressing transcription (Vignais et al., 1987; Warner, 1989), are required for initiation of transcription of most yeast r-protein genes. Tandem Rap1 binding sites, or a single autonomously replicating sequence binding factor (Afb1) binding site (Planta et al., 1995) upstream of a T-rich region that enhances transcription, comprise the *cis* elements present in most promoters of yeast r-protein genes. R-protein gene expression is enhanced by Esa1-driven histone acetylation and is mediated by Afb1 and Rap1 activity (Reid et al., 2000). The target of rapamycin (TOR) pathway can also regulate r-protein gene expression (Powers and Walter, 1999; Warner, 1999). The serine/threonine kinases TOR1 and TOR2 activate r-protein gene

expression in the presence of abundant nutrients and TOR signaling is a requirement for efficient Esa1 mediated acetylation of r-protein gene associated histones (Rohde and Cardenas, 2003). Forkhead-like1 (Fhl1: forkhead DNA-binding domain containing transcription factor) can stimulate r-protein gene expression through both TOR and TOR independent channels and its activity is enhanced through an interaction with Rap1 (Lee et al., 2002; Wade et al., 2004).

#### **1.5.2.3.2 Animal R-Protein Gene Regulation**

In contrast to both prokaryotic and yeast r-protein gene regulation, little is known about animal and plant r-protein gene regulation. Primarily animal r-protein gene expression is regulated translationally, with secondary regulation at the levels of transcription and posttranscription and regulation is intimately coordinated with growth and development (Mager, 1988; Larson et al., 1991). R-protein mRNAs alternate between inactive subpolysomal (mRNPs) fractions and active polysomes, depending on the activity of a cell (Meyuhas, 2000). In both *Xenopus* and mammals, 70-85% of r-protein mRNAs are associated with polysomes in active cells while in resting (or inactive) cells this drops to 20-50% (Geyer et al., 1982; Aloni et al., 1992). A key feature found in the 5' UTR of most vertebrate r-protein mRNAs is the 5'-TOP motif at the transcription start site. The TOP is comprised of a C residue at the 5' cap site followed by a stretch of pyrimidines (4-14 in mammals and 8-12 in *Xenopus*), followed by a GC-rich region. Although present in most vertebrate and some invertebrate r-protein genes, the mechanism behind 5'-TOP motif-mediated regulation of translational of r-protein mRNAs is not fully understood (Amaldi et al., 1995; Meyuhas, 2000; McIntosh and Bonham-Smith, 2006).

TATA boxes, consensus initiator sequences ((Y)<sub>2</sub>C<sup>+</sup>TY(T)<sub>2</sub>(Y)<sub>3</sub>) located within polypyrimidine tracts and consensus motifs for Yin Yang 1 (YY1: member of the GL1-krüppel family of zinc-finger transcription factors that interacts with histone acetyltransferases and histones deacetylases) and GA-binding protein (GABP: heterodimeric transcription factor) are all common in animal r-protein gene regulatory regions (Thomas and Seto, 1999; Perry, 2005). Like Afb1 and Rap1 in yeast, YY1 and GABP are r-protein specific transcriptional repressors and activators and are not found in any other TOP-containing genes (Thomas and Seto, 1999; Perry, 2005). Animal r-protein

introns can also be important in transcriptional and posttranscriptional regulation of gene expression. Introns in the 5' UTR of r-protein genes can physically separate the regulatory region from the coding region and/or contain sites for YY1 transcription factors, and subsequent transcriptional regulation (Amaldi et al., 1995; McIntosh and Bonham-Smith, 2006). The presence of introns can effect posttranscriptional gene regulation through alternative splicing (cell-specific regulation) generating alternative transcripts or transcripts with premature termination codons that will be targeted for degradation (Mitrovich and Anderson, 2000; McIntosh and Bonham-Smith, 2006).

#### **1.5.2.3.3 Plant R-Protein Gene Regulation**

Most current plant r-protein research has been restricted to the characterization of developmental/stress/tissue transcript expression profiles of the different r-protein family members. Some members of r-protein families are differentially expressed in response to a variety of factors including developmental stage, tissue type and stress (i.e. mechanical wounding, auxin, cytokinin and abscisic acid treatment, UV-B radiation, cold and heat shock and avirulent pathogen attack resulting in the hypersensitive response), suggesting the presence of some level of transcriptional regulation (McIntosh and Bonham-Smith, 2006). However, as would be expected from limited investigation, few gene regulatory motifs have been identified in plant r-protein genes, although some elements similar to those in r-protein genes of other (more thoroughly investigated) organisms have been identified. In comparison to r-protein transcripts of other eukaryotic organisms, the 5' UTRs of plant r-protein transcripts are of similar length and content in that they are only several hundred nucleotides long and contain several common *cis* elements including a TATA box, *telo* box, *tef* box and site II motif (McIntosh and Bonham-Smith, 2006). *telo* boxes (5'-AAACCCTA-3'), a common *cis* element in plant r-protein genes (found in 174 of 216 Arabidopsis upstream regulatory regions), are located within 55 base pairs of a TATA box, usually as an inverted repeat (Moran, 2000) and in concert with *tef* boxes and acidic r-protein *trap40* boxes, are required for root primordia r-protein gene expression (Lenvik et al., 1994; Manevski et al., 2000; Tremousaygue et al., 2003). The *tef* box (5'-ARGGRYAnnnnnGTM-3': R = purine, Y = pyrimidine, M = A or C) is commonly found along with a *telo* box in upstream regulatory regions but unlike the *telo* box, can initiate gene expression without the presence of other



*cis* elements (Regad et al., 1995). Site II motifs (*PROLIFERATING CELL NUCLEAR ANITGEN* (*PCNA*) site II motif: 5'-TGGGCC/T-3'), have been identified in 88% of *telo* box containing plant r-protein genes and in conjunction with the *telo* box, stimulates gene expression in mitotically active tissues (Tremousaygue et al., 2003). A site II motif is always located upstream of a *telo* box and regulates expression of r-proteins most commonly in young leaves and root primordia, while the *telo* box enhances the levels of expression in these tissues (Tremousaygue et al., 2003; McIntosh and Bonham-Smith, 2006).

Transcriptional, posttranscriptional, translational and posttranslational gene regulation of plant r-protein genes remains poorly understood, however, some form of translational regulation has been suggested in Arabidopsis. Following a drought stress, r-protein transcripts are found in lower abundance in polysomes, similar to the translational regulation of expression of r-protein mRNAs in animal resting cells (Geyer et al., 1982; Aloni et al., 1992; Kawaguchi et al., 2004). In response to heat (tomato culture and maize embryos) and hypoxic (maize seedling roots) stresses, RPS6 was shown to be dephosphorylated resulting in decreased ribosome biogenesis and global translation rate (Bailey-Serres et al.; Scharf and Nover, 1982; Beltran-Pena et al., 2002). Removal of the stress resulted in rephosphorylation of RPS6 and a return to normal translational status, illustrating the profound effect of the phosphorylation status of a single r-protein on global translation (Scharf and Nover, 1982; Turck et al., 2004).

## **1.6. Extraribosomal Functions**

In addition to their recognized ribosomal functions, many r-proteins also exhibit extraribosomal functions. To function outside of their recognized ribosomal roles may be an attribute to the evolutionary origins of these r-proteins. Two theories prevail: (1) r-proteins evolved specifically for use in ribosomes and were later adapted to roles outside of the ribosome or, (2) proteins with predefined functions have been adopted and adapted to the ribosome to increase efficiency and/or specificity of the ribosome in a variety of situations (Wool, 1996). The presence of nucleic acid binding domains in r-proteins (zinc finger, bZIP and helix-turn-helix motifs) suggests that early adopted r-proteins to the ribosome catalytic rRNA may have previously had the capacity to bind DNA.

As a transcriptional regulator in humans, RPS3 binds through a K homology (KH)-domain, to the NF- $\kappa$ B p65 homo-dimer and the p65-p50 hetero-dimer to enhance DNA binding of these complexes. Any mutation of RPS3 has been shown to decrease the ability of NF- $\kappa$ B to stimulate downstream gene expression (Wan et al., 2007). In addition to its role as a transcriptional regulator, upon DNA damage, RPS3 can be phosphorylated and imported into the nucleus where, in its role as a DNA repair endonuclease, it is involved in excision repair of damaged base pairs (Yadavilli et al., 2007) or it can stimulate caspase-dependent apoptosis (Jang et al., 2004).

In mammals, RPL11 can indirectly influence the cell cycle and tumorigenesis by binding to the oncoprotein Myc and inhibiting its ability to bind and activate its target genes (Dai et al., 2007). Miz1 is a Myc-associated zinc-finger protein required to arrest cell cycle proliferation, thus preventing tumorigenesis. R-proteins have been shown to be essential for Miz1-dependent transactivation. RPL23 indirectly activates Miz1 by retaining the Miz1 activator B23 (NPM/nucleophosmin) in the nucleolus (Neumann and Krawinkel, 1997).

Effectors of nucleolar/ribosomal stress, such as serum and nucleotide starvation, treatment with ribosome assembly inhibitors such as actinomycin D or 5-Fluorouracil, or mutations in genes that encode proteins essential for ribosome biogenesis (including many r-proteins), all induce cell cycle arrest (Zhang and Lu, 2009). Upon nucleolar disruption, r-proteins no longer shuttle from the cytoplasm through the nucleus to the nucleolus for LSU and SSU assembly. In mammals, a growing number of r-proteins, including RPL5, RPL7, RPL11, RPL23, RPL26, RPL29, RPL30, RPL37, RPS3, RPS6, RPS7, RPS9 and RPS27 can indirectly influence the cell cycle through interactions with the p53-MDM2 (E3 ubiquitin ligase murine double minute 2 protein (HDM2 in humans)) pathway (Chen et al., 2007; Horn and Vousden, 2008; Ofir-Rosenfeld et al., 2008; Fumagalli et al., 2009; Yadavilli et al., 2009; Zhang and Lu, 2009; Zhu et al., 2009; Daftuar et al., 2010; Xiong et al., 2011). MDM2 negatively regulates the transcription factor p53 that has a range of target genes involved in apoptosis, cell cycle progression, senescence, differentiation, DNA repair, miRNA processing and cellular metabolism (Bernstein et al., 2007; Kruse and Gu, 2009; Suzuki et al., 2009; Vousden and Ryan, 2009). Under normal physiological conditions, MDM2 binds

to and targets p53 for degradation via the 26S proteasome, thus blocking its ability to function as a transcription factor (Lohrum et al., 2003; Zhang and Lu, 2009). Following nucleolar stress, free r-proteins released into the nucleoplasm from the nucleolus bind to the acidic domain and sometimes the C-terminal domain of MDM2, thereby, interfering with its E3 ubiquitin ligase activity. As a result of this decrease in functional MDM2, p53 becomes stabilized and abrogates cell cycle progression (Horn and Vousden, 2008; Ofir-Rosenfeld et al., 2008). Interestingly, r-protein deficiency does not always result in p53 stabilization. While overexpression of RPS29, RPL13a and RPS27 promote p53 stabilization, deficiencies in RPS9, RPS13, RPL13 and RPL35a inhibit p53, highlighting the need for further investigation (Chen and Ioannou, 1999; Lopez et al., 2002; Khanna et al., 2003; Kim et al., 2003; Shi et al., 2004; He and Sun, 2007).

In humans, RPL13a can also function as a translational repressor of *CERULOPLASMIN* mRNA. Phosphorylated RPL13a binds the 3' UTR of interferon-Gamma-Activated Inhibitor of Translation (GAIT) element of *CERULOPLASMIN* mRNA in the presence of Interferon- $\gamma$ , resulting in repression of translation of this mRNA (Mazumder et al., 2003). Also in humans, RPL7 and RPS13 have been shown to regulate translation of their own mRNA and in the case of RPL7, several other nuclear encoded mRNAs (Neumann et al., 1995).

RPL26 emphasizes the diverse roles r-proteins can possess in regulating the cell cycle through the p53-MDM2 pathway. Under non-stress conditions, RPL26 can be bound by MDM2, and be polyubiquitinated for subsequent proteasome-mediated degradation (Bernstein et al., 2007). This degradation of RPL26 ensures low levels of p53 and that the cell cycle will proceed unhindered. When a stress is perceived, MDM2 is post-translationally modified to decrease its affinity for RPL26, resulting in increased RPL26-p53 mRNA binding and subsequent p53 translation (Takagi et al., 2005; Bernstein et al., 2007). Upon DNA damage, RPL26 can bind the dsRNA UTR (5' and 3' UTRs fold back upon themselves to form a dsRNA UTR) of p53 mRNA, increasing its affinity for polysomes and thus acting as a positive regulator of translation of p53 mRNA (Takagi et al., 2005). The ability to regulate translation of p53 mRNA sets RPL26 apart from many r-proteins whose

sole role is that of MDM2 inhibition, thus, highlighting the complexities of r-protein roles away from the ribosome.

### 1.7. Ribosomal Protein Mutants

A variety of r-protein mutants have been identified in a variety of organisms and are generally characterized by deleterious phenotypes in the host. In plants, *rpl23aa*, *rps5b*, *rps13b*, *rps18a* and *rpl24b* all share a similar range of phenotypes. Degenhardt and Bonham-Smith (2008) reported the pleiotropic phenotypes associated with *rpl23aa* in *Arabidopsis* that included delayed flowering, aberrant leaf venation, abnormal root morphology, loss of apical dominance and production of the *pointed first leaf* phenotype (*pfl*: analogous to *minute* in *Drosophila melanogaster*) (Lambertsson, 1998; Degenhardt and Bonham-Smith, 2008).

Mutations in *RPS18A* and *RPS13A* (Vanlijsebettens et al., 1994; Ito et al., 2000) confer a similar phenotype (aberrant growth and development) to those found in *rpl23aa*, whereas, *RPS27A* disruption does not produce the *pfl* phenotype, but does exhibit similar abnormal root morphology as *rpl23aa* mutants (Revenkova et al., 1999; Degenhardt and Bonham-Smith, 2008). Roots in *rpl27a* and *rpl23aa* are characterized by short root hairs and abnormal lateral roots (Revenkova et al., 1999; Degenhardt and Bonham-Smith, 2008). A T-DNA insertion in the *RPS5A* dominant paralog of the *Arabidopsis* two-member *RPS5* gene family is homozygous lethal and shows delayed cell division in heterozygous lines (Weijers et al., 2001). In depth phenotypic analysis of *rpl24b* revealed a weak *pfl* phenotype as well as short siliques, reduced fertility, abnormal ovule development, retarded above and below ground growth and a reduced growth rate (Nishimura et al., 2005). Transposon insertional mutagenesis of *RPS16* produced a lethal phenotype resulting from arrested development during the globular to heart stage transition of the embryo (Tsugeki et al., 1996). Similar r-protein mutant phenotypes have been identified in *Nicotiana tabacum*, e.g. *rpl3b* shows delayed development, reduced lateral root growth and abnormal cell division, all phenotypes associated with *Arabidopsis* r-protein mutants (Popescu and Tumer, 2004).

Delayed growth and development are not specific to plant r-protein mutants, with similar phenotypes found in human, insect, yeast and mice r-protein mutants. The *minute*

phenotype in *Drosophila melanogaster* was the first phenotype attributed to a reduction in r-protein expression (Kongsuwan et al., 1985). *minute* flies exhibit short thin bristles, slow development, reduced viability, rough eyes and small body size. A complementation with *RPL49* rescued the 99D (*rpl49*) *minute* mutant, however discrete *minute* mutants were unaffected by the introduction *RPL49*, confirming in this case, the specificity of the phenotype to a disruption in *RPL49* (Kongsuwan et al., 1985).

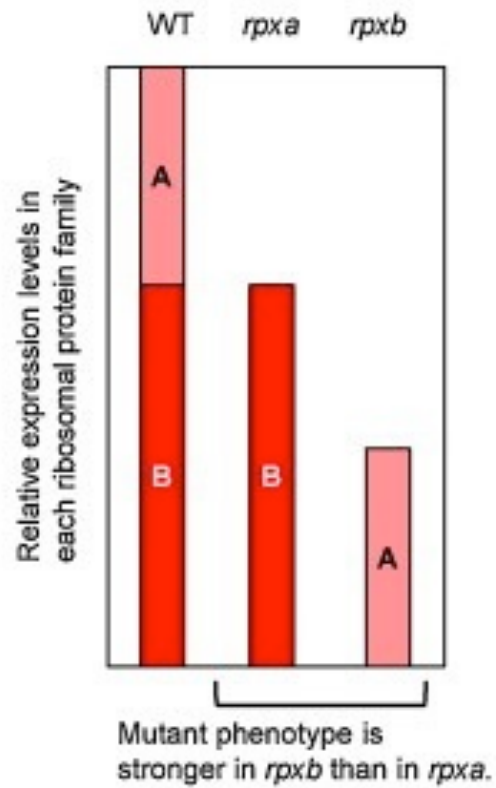
A knockout of *RPS6* in mice liver cells prevented these cells from progressing through the cell cycle, possibly due to reduced cyclin E expression (cyclin E activates cyclin dependent kinase 2 which is required for a cell to enter into the S phase) (Geng et al., 2007). Although the cells could no longer progress through the cell cycle and thus not proliferate, they were still able to synthesize proteins and grow, indicating specificity for a disruption in cell cycle progression and not ribosome biogenesis (Volarević et al., 2000; Geng et al., 2007). The haploinsufficient genetic disorder, Turner syndrome, results in a variety of growth defects including infertility and reduced stature. These phenotypes have been attributed to some degree to the loss of a functional X-linked *RPS4* during early female development (Warner and Nierras, 1998; Zinn and Ross, 1998; Barakat et al., 2001).

## **1.8. Ribosomes and Development**

Many plant r-protein mutants exhibit rare developmental phenotypes (as described in section 1.7), suggesting that functional ribosomes and efficient translational regulation are essential for normal development (Horiguchi et al., 2012). Horiguchi and colleagues (2012) have proposed three models; ribosome insufficiency, heterogeneity and aberrancy to explain how ribosomes may be involved in plant growth and development.

### **1.8.1. Ribosome Insufficiency Model**

The ribosome insufficiency model was proposed to account for the similar phenotypes associated with the different r-protein mutants. The model proposes that the lack of an individual r-protein reduces the general availability of ribosomes and that the resulting ribosomes do not function efficiently or are rapidly degraded. The ribosome insufficiency model proposes that for example, in a two-member gene family if both paralogs are required for normal growth, the degree of insufficiency would depend on the

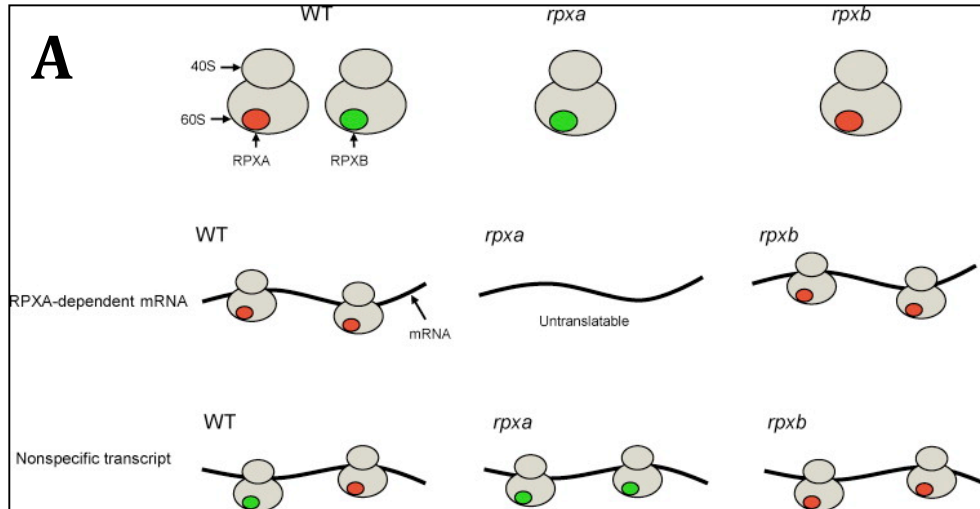


**Figure 1.2: Ribosome insufficiency model.** Phenotypes associated with r-protein mutants are dependent on the relative expression levels of each paralog. More severe phenotypes would be associated with the more dominant paralog (Taken with permission from Horiguchi et al., (2012)).

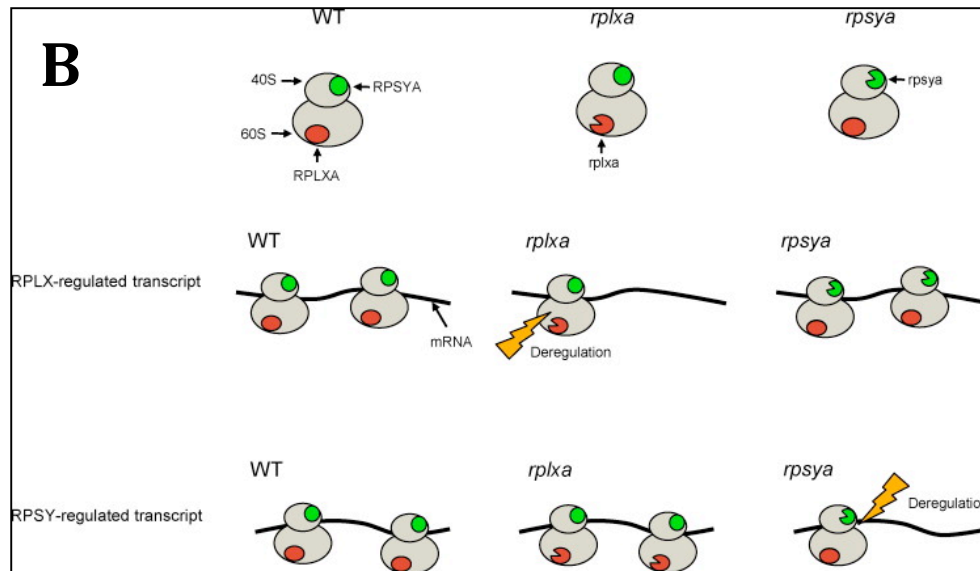
relative expression of each of the two paralogs (Figure 1.2). In the Arabidopsis two-member *RPS5* gene family, *RPS5A* expression is much greater than *RPS5B*, therefore, *rps5a* exhibits a dominant mutant phenotype (Weijers et al., 2001). Supporting this model, polysome profiles of *rps6a* and *rps6b* single mutants show a reduced number of 40S subunits, with a concomitant increase in 60S subunits, compared to 80S ribosomes (Creff et al., 2010). Also, global protein synthesis was decreased in *rpl4a* and *rpl4d* single mutants compared to WT (Rosado et al., 2010). Although mutations in these r-proteins leads to decreased translation, this outcome could also be due to structurally/functionally abnormal ribosomes rather than decreased ribosome numbers.

### 1.8.2. Ribosome Heterogeneity Model

The ribosome heterogeneity model proposes that the multiple expressed paralogs of each gene family are required for mRNA species-specific translation (Figure 1.3A) (Horiguchi et al., 2012). In support of this model, 59 of 79 yeast cytosolic r-proteins are encoded by two member gene families and the translation of *ASYMMETRIC SYNTHESIS OF HO 1* requires the paralogs *RPL7A*, *RPL12B*, *RPL22A* and *RPS18B* but not *RPL7B*, *RPL12A*, *RPL22B* or *RPS18B* (Komili et al., 2007). In plants, no clear examples of r-protein paralog-specific mRNA translation have been reported, however, two promising r-protein family candidates are the *RPL5* and *RPL10* gene families. While producing near identical single mutant phenotypes in true leaves, Arabidopsis *rpl5a* embryos/seedlings had one to four cotyledons compared to *rpl5b*, that only had two, suggesting a role in embryo development that is specific to *RPL5A* (Fujikura et al., 2009). The Arabidopsis *RPL10* gene family contains three members that are differentially expressed. Homozygous *rpl10a* was lethal while heterozygous plants showed no abnormal phenotypes (Ferreyra et al., 2010). Homozygous *rpl10b* plants exhibited the *pfl* phenotype while homozygous *rpl10c* plants, similar to heterozygous *rpl10a* plants, showed no developmental abnormalities (Ferreyra et al., 2010). Interestingly, only two of the three members were shown to be UV regulated. *RPL10B* was down regulated upon UV-B exposure while *RPL10C* was upregulated. These results suggest that *RPL10A/B* and *C* are not functionally redundant in Arabidopsis



## Ribosome Heterogeneity



## Ribosome Aberrancy

**Figure 1.3. Ribosome heterogeneity and aberrancy models.** (A) Ribosome heterogeneity model. Translation is dependent on individual paralogs from each family, therefore, paralogs are not functionally redundant. WT populations of ribosomes (no mutations in either *RPXA* or *RPXB*: hypothetical r-protein genes from a two member *RPX* gene family) have the capacity to translate any mRNA species. *RPXA* dependent mRNA translation is unable to occur in the *rpxa* mutant but still occurs in the *rpxb* mutant. Translation is dependent on the *rpxa* paralog, not *rpxb*. (B) Ribosome aberrancy model, where gene family members are functionally redundant. The absence of a protein (ie. RPLXA or RPSYA) results in aberrant ribosomes and translation deregulation of the dependent mRNA species (Taken with permission from Horiguchi et al., (2012)).



(Ferreyra et al., 2010). Until transcript expression patterns for a variety of small and large subunit, including *RPL5* and *RPL10*, gene families are determined, conclusions concerning the proposed ribosome heterogeneity model cannot be made (Horiguchi et al., 2012).

### 1.8.3. Ribosome Aberrancy Model

The final model proposed by Horiguchi and colleagues (2012) is the ribosome aberrancy model. This model assumes all members of the same family are functionally equivalent and aberrant r-protein isoforms are incorporated into ribosomes, resulting in aberrant ribosomes effecting translation in a paralog specific manner (Horiguchi et al., 2012). The model proposes that a mutation in any one paralog from an individual r-protein gene family will affect gene family-specific mRNA species-specific translation (Figure 1.3B). This is most evident when looking at r-protein:*asymmetric leaves 2 (as2)* double mutants. *rpl4d* in the *as2* background strongly influences leaf dorsoventral polarity but weakly influences cell proliferation while *rps28b:as2* plants show a strong effect on cell proliferation and weak effect on dorsoventral leaf polarity (Horiguchi et al., 2011).

It is important to view these models not as separate processes of which only one maybe responsible for a resulting phenotype but as a complex and dynamic combination of all three contributing to the final mutant phenotype (Horiguchi et al., 2012).

## 1.9. *Arabidopsis thaliana* RPS15a Gene Family

RPS15a is the eukaryotic ortholog of prokaryotic RPS8 and is highly conserved among all three domains of life. Plant *RPS15a* was initially identified in *Brassica napus* and later in *Arabidopsis* by comparison to rat and yeast *RPS15a* (Bonham-Smith et al., 1992). In *Arabidopsis*, six members encode the *RPS15a* gene family, *RPS15aA-F*, with one member, *RPS15aC*, not transcriptionally active (Hulm et al., 2005). The five active members encode four isoforms (*RPS15aA* and *-F* are identical, *RPS15aD*, *RPS15aB* and *RPS15aE*). These six members can be divided into two evolutionary distinct groups, type I (*RPS15aA*, *C*, *D* and *F*) and type II (*RPS15aB* and *E*). Type I members clade with rat S15a (73.1 – 77.7% amino acid sequence similarity; *RPS15aA/F* and *D* respectively), *Drosophila* and yeast, while type II members form a separate clade, along with *Oryza sativa* S15a2 and *Zea mays* S15a2, and

exhibit much lower amino acid sequence similarity to rat S15a (47.6 and 48.8%; RPS15aB and E, respectively) (Barakat et al., 2001; Chang et al., 2005). Supporting evidence for this classification system comes from localization assays performed in the Bonham-Smith lab. RPS15aA/F and D isoforms all localize to the nucleus/nucleolus while RPS15aB and E exhibit non-nuclear/nucleolar or mitochondrial localization (Hulm et al., 2008; Chapter 2). Cytosolic (type I) *RPS15a* genes have been well studied and are highly conserved among plants and animals while the non-cytosolic (type II) *RPS15a* genes have remained relatively unstudied and to date, appear to be plant specific.

The six genes comprising the Arabidopsis *RPS15a* gene family are located on all five chromosomes: *RPS15aA* (I), *RPS15aB* and *C* (II), *RPS15aD* (III), *RPS15aE* (IV) and *RPS15aF* (V). Type I genes (*RPS15aA*, *C*, *D* and *F*) all contain three exons and two introns and exhibit nucleotide sequence similarity ranging from 75 – 90% in the open reading frames (ORFs) and 87 – 100% in the proteins, with RPS15aA and F encoding identical proteins (Hulm et al., 2005). WT transcript expression of type I *RPS15a* paralogs (except *RPS15aC*) is highest in mitotically active tissues (apical meristems, young leaves and flower buds) and lowest in mature tissue such as cauline leaves and bracts (Hulm et al., 2005). Treatment of seedlings with BAP resulted in increased expression of all cytosolic members while IAA treatment specifically increased *RPS15aF* transcript levels. ABA application decreased expression of all cytosolic members while GA3 treatment had little effect on any family members (Hulm et al., 2005). Expression of type I members were little effected by heat stress, cold acclimation or wounding. Putative regulatory elements in the 1000 bp upstream of the ATG start codons of the active type I members were identified as a root specific element (5'-ATATT-3'), two auxin response elements (5'-TGACG-3' and 5'-ACTTTA-3') and a low temperature responsive element (5'-CCGAAA-3': specific to *RPS15aF*), together with *tef*, *telo* and Site II motifs (Hulm et al., 2005).

### **1.10. Objectives**

In this thesis, I have examined how reduced translational efficiency (due to decreased ribosome biogenesis) results in the phenotypes associated with r-protein, micro RNA biogenesis and auxin synthesis mutants. The sub-cellular locations of the proteins

encoded by the *RPS15a*, *RPS13*, *RPS18* and *RPL18* gene families were also determined. The specific objectives of my thesis were:

- 1) Determine individual and family requirements of *RPS15a* isoforms for normal plant growth and development.
- 2) Determine the subcellular localization of r-protein isoforms encoded by the *RPS15a*, *RPS13*, *RPS18* and *RPL18* gene families.
- 3) Identify transcriptome and miRNOME changes and similarities in the *pointed first leaf* mutants *pfl1* (*rps18a*) and *pfl2* (*rps13a*).
- 4) Identify up or down regulated genes common to r-protein and auxin mutants.
- 5) Document a novel *rps18a:HF/RPL18B* mutant in Arabidopsis.

## CHAPTER 2. CHARACTERIZING THE *RPS15A* GENE FAMILY

*RPS15a* is a novel Arabidopsis r-protein gene family in that it contains both cytosolic (type I: *RPS15aA/C/D* and *F*) and non-cytosolic (type II: *RPS15aB* and *E*) ribosomal constituents, whereas most contain only cytosolic members. C-terminal truncations of *RPS15aA/F* and *D*, including the removal of a C-terminal pentapeptide motif, demonstrated that nuclear/nucleolar localization required the last seven C-terminal amino acids of each isoform. These deletions may affect proper protein folding (required for normal nuclear (NLS) and/or nucleolar (NoLS) localization signaling) or r-protein nuclear pore complex (NPC) receptor interactions, thereby, preventing r-protein entry into the nucleus.

*RPS15aB* and *E* have been less definitively characterized. Here using confocal microscopy I show that *RPS15aB* and *E* are not localized to, 1) the nucleus or nucleolus (like their type I cytosolic counterparts) or, 2) mitochondria, as previously suggested. Under a variety of experimental conditions only weak cytoplasmic localization was recorded for *RPS15aB* and *E*. Although cellular location is presently unknown, an RNAi-mediated family knockdown (KD) of *RPS15aB* and *E* (type II members) indicated that at least one member is required for normal plant growth and development, while both type I and type II family KDs were lethal. Individual type I or II gene KD lines were inconclusive. As several r-proteins are recruited to the ribosomal SSU and LSU following export from the nucleolus to the cytoplasm, an antibody was generated against a *RPS15aE* peptide to monitor the presence/absence of *RPS15aB* and *E* in seedling total protein fractions. No *RPS15aB* or *E* was identified in the total protein fraction by LC-MS/MS.

### 2.1. Introduction

In Arabidopsis, r-protein gene families consist of two to seven members, most of which are transcriptionally active, resulting in different protein isoforms incorporated into different ribosomes (Chang et al., 2005). Regulation of r-protein genes is poorly understood but it has been shown that under varying environmental and/or developmental pressures, expression of different members of each gene family is independently up or down regulated. In the small subunit gene family *RPS15a*, there are six members, of which only five are actively transcribed (Hulm et al., 2005) and incorporated into functional ribosomes (Chang et al., 2005). *RPS15a* is thought to be a

primary 18S rRNA binding protein, absolutely required for ribosome assembly as experimentally determined through its eubacterial ortholog RPS8 (Held et al., 1974; Svensson et al., 1988; Wool, 1996; Nevskaya et al., 1998; Adams et al., 2002). Based on sequence similarity, the Arabidopsis *RPS15a* gene family can be divided into two types. Type I (cytosolic) members are *RPS15aA/C/D* and *F* where *RPS15aC* is an inactive pseudogene and *RPS15aA* and *F* encode identical proteins (Figure 2.1). Type II (non-cytosolic – may be referred to as mitochondrial in the literature) members are *RPS15aB* and *E*. Type I members show similarity to other eukaryotic sequences including *Rattus norvegicus*, *Drosophila* and yeast while the plant specific type II Arabidopsis *RPS15as* clade with maize and rice *RPS15a2s* (Chang et al., 2005). In Arabidopsis cytoplasmic ribosomes, type I RPS15a isoforms were found in higher abundance than the more acidic type II proteins (Chang et al., 2005). Type I isoforms share 75 – 90% nucleotide sequence identity among coding sequences but only 46 – 49% identity among their 5' UTRs despite containing many of the same regulatory motifs (Hulm et al., 2005). Hulm et al., (2005) further demonstrated that the three active type I members are regulated slightly differently in response to a variety of stimuli; 6-benzylaminopurine (BAP), indole acetic acid (IAA), abscisic acid (ABA), gibberellic acid (GA<sub>3</sub>) and mechanical stress.

The role of the plant specific type II RPS15a isoforms is still unknown, as their location in the cell is still uncertain. In 2002, Adams and co-workers proposed that in angiosperms, RPS15aB and E were compensating for the loss of mitochondrial RPS8. With no identified nuclear gene for mitochondrial RPS8, it was hypothesized that *RPS8* was lost from the mitochondrial genome altogether and to compensate for this loss the two nuclear genome located genes, *RPS15aB* and *E*, acquired mitochondrial localization sequences and have functionally replaced RPS8 in the mitochondria. This theory has some support: (1) in an *in vitro* assay, RPS15aB and E isolated from Arabidopsis and tomato were successfully imported into soybean mitochondria (Adams et al., 2002) and (2) three independent mitochondrial protein prediction programs (Mitoprot, TargetP and Predotar) have all identified RPS15aB and E as putative mitochondrial proteins (Carroll et al., 2008). While

RPS15aA	ATGGTAAGAATCAGTGTTCCTTAACGATGCTCTCAAGAGCAT-GTACAATGCTGAGAAACG	59
RPS15aD	ATGGTGAGAATCAGTGTGCTCAATGATGGTCTTAAGAGTAT-GTACAATGCAGAGAAGAG	59
RPS15aF	ATGGTGAGAATCAGTGTGCTTAACGATGCTCTTAAGAGTAT-GTACAATGCTGAGAAGAG	59
RPS15aB	ATGGGGAGG---AGAATATTGAACGATGCATTGAGAACGATCGTG-AATGCTGAGAAGCG	56
RPS15aE	ATGGGGAGG---AGGATTTTGAACGATGCGTTGAGGACGATTGTG-AATGCTGAGAGACG	56
	**** ** * * * * * * * * * * * * * * * *	
RPS15aA	AGGCAAGAGGCAGGTCATGATCAGGCCCTTCCTCCAAAGTGATTATCAAGTTTCTGATTGT	119
RPS15aD	AGGAAAGAGGCAGGTTATGATCAGGCCCTTCCTCTAAAGTTATCATCAAGTTCTTATCGT	119
RPS15aF	AGGGAAGAGGCAAGTCATGATCAGGCCCTTCCTCTAAAGTGATCATCAAGTTTCTTATCGT	119
RPS15aB	AGGGAAGAGCTTCGGTGGAGTTGAAACCTGTCCTACCGTAATGCTTCGTTTCTCAAAAT	116
RPS15aE	AGGAAAGAGCTTCGGTGGAGCTCAAACCAATCTCCACCGTTATGCTTCGTTCTTGAGAAT	116
	*** ** * * * * * * * * * * * * * * *	
RPS15aA	CATGCAGAAGCACGGTTACATTGGTGAGTTT-GAGTATGTCGATGACCACAGATCTGGCA	178
RPS15aD	CATGCAAAAGCACGGTTACATTGGCGAGTTT-GAGTATGTTGATGACCACAGGTCGGTA	178
RPS15aF	CATGCAGAAACACGGTTACATTGGTGAGTTT-GAGTACGTTGATGACCACAGGTCGGAA	178
RPS15aB	CATGAAAGAGAAAGGGTACATCAAGAACCTTCCAAGTCCAT-GATCCACACAGAGTTGGAA	175
RPS15aE	CATGAAGGAGAAAGGTATATCAAGAACCTTCCAAGTGTAT-GATCCACATAGAGTTGGGA	175
	**** * * * * * * * * * * * * * * *	
RPS15aA	AGATCGTCGTTGAATTGAACGGAAGGTGAACAAGTGTGGTGTATCAGCCCACGTTTGT	238
RPS15aD	AGATTGTTGTTGAGCTTAATGGAAGGCTGAACAAGTGTGGAGTTATCAGTCCTCGTTTCG	238
RPS15aF	AGATCGTGGTCGAGCTTAATGGAAGACTTAACAAATGTGGAGTCATCAGTCCTCGTTTCG	238
RPS15aB	GAATAACTGTTGATCTACAAGGAAGGGTTAATGATTGCAAAGCTCTCATTATAGGCAGG	235
RPS15aE	GAATAACAGTTGATCTGCAAGGGAGGGTTAATGACTGCAAAGCTCTTACCTACAGGCAAG	235
	** * * * * * * * * * * * * * * *	
RPS15aA	ATGTTGGTGTCAAGGAAATTGAAGGTTGGACTGCCCGTTTGCTCCCTTCTAGACAGTTTG	298
RPS15aD	ATGTCGGTGTTAAGGAGATTGAAGGATGGACTGCTCGTCTACTTCCCTTCCAGACAGTTTG	298
RPS15aF	ATGTTGGAGTTAAGGAGATTGAAGGCTGGACCGTCGTCTTCTTCCCTTCCAGACAGTTTG	298
RPS15aB	ACGTTAAGGCAAAATGAGATCGGGCAATACACAGAACGCACACTTCCAACGCGCCAGTGGG	295
RPS15aE	ACGTTAGGGCAAAAGAGATTGAGAAATACACTGAACGCACACTTCCAACACGTCAAGTGGG	295
	* * * * * * * * * * * * * * * *	
RPS15aA	GTTACATCGTGCTGACTACATCTGCTGGCATTATGGACCATGAAGAAGCTAGAAGGAAGA	358
RPS15aD	GTTTCATTGTTCTGACGACCTCAGCGGGAATCATGGATCATGAAGAAGCTAGGAGAAAGA	358
RPS15aF	GGTACATTGTTCTTACGACCTCGGCTGGTATTATGGACCACGAAGAAGCCAGGAGAAAGA	358
RPS15aB	GTTATATAGTGATTACAACCTCCCGATGGCATTGTTGGATCATGAAGAAGCTATTAAACGGA	355
RPS15aE	GTTATGTTGTAATCAACCTCCCGATGGGATTTTGGACCATGAAGAAGCAATCAAACGGA	355
	* * * * * * * * * * * * * * * *	
RPS15aA	ATGTTGGAGGCAAGGTTCTCGGCTTCTTCTATTGA	393
RPS15aD	ACGTTGGTGGCAAAGTTCTTGGGTTCTTTTACTGA	393
RPS15aF	ATGTTGGTGGCAAAGTTCCTTGGCTTCTTTTACTGA	393
RPS15aB	ATGTGGGTGGTCAGGTTCTTGGTTTCTTTTACTGA	390
RPS15aE	ATGTGGGTGGTCAGGTTCTTGGCTTCTTTTACTGA	390
	* * * * * * * * * * * * * * *	

**Figure 2.1. Coding sequence alignment of type I and II *RPS15a* family members.**

The *RPS15a* family is comprised of six members of which five are transcribed and translated into r-proteins that are incorporated into functional ribosomes (*RPS15aC* is a pseudogene). Type I members are *RPS15aA/D* and *F* and type II members are *RPS15aB* and *E*. Yellow sequence – conserved among type I members; turquoise sequence – conserved among type II members; asterisk – conserved among all five actively transcribed members of *RPS15a*.

Chang et al. (2005) initially associated RPS15aB and E with cytoplasmic ribosomes, as determined by LC-MS/MS, Carroll and co-workers (2008) again using LC-MS/MS showed RPS15aB and E to be associated with samples enriched for mitochondrial ribosomes and not cytosolic ribosomes. In contrast, type I isoforms (RPS15aA/D and F) were only found in cytoplasmic fractions and not mitochondrial ribosome samples. At the same time it was suggested that the RPS15aB/E cytoplasmic ribosome association suggested by Chang et al. (2005) may have resulted from mitochondrial contamination of the cytoplasmic fraction and that improved isolation techniques resulted in a more accurate mitochondrial localization of the RPS15a type I and type II isoforms in *Arabidopsis* (Carroll et al., 2008).

Most cytoplasmic r-proteins, after synthesis in the cytoplasm, are transported through the nucleus to the nucleolus for large and small subunit assembly. Transport into the nucleus is mediated through nuclear pore complexes (NPCs), large 40 – 125 MDa multiprotein complexes comprised of approximately 30 nucleoporin proteins (Cronshaw et al., 2002; Lim et al., 2008). NPC-mediated transport of proteins into the nucleus requires the presence of nuclear localization signals (NLSs) in the cargo protein. NLSs are generally comprised of one (classical/canonical monopartite NLS) or more (bipartite NLS) stretches of basic amino acids (lysine (K) and arginine (R)) that interact with the acidic importin/karyopherin family of proteins comprising part of the NPC (Dingwall et al., 1988; Chelsky et al., 1989; Chook and Blobel, 2001; Mosammaparast and Pemberton, 2004; Pemberton and Paschal, 2005). Nucleolar localization signals (NoLSs) are much harder to identify as disruption of nuclear localization often by definition results in disrupted nucleolar localization. Most of our understanding of NoLSs has come from human nucleolar proteins. Generally a NoLS is between seven to thirty amino acids long, predominantly comprised of Ks and Rs and may be distinct to or part of a NLS (Sheng et al., 2004; Emmott and Hiscox, 2009). Once located in the nucleolus, an r-protein may be retained in this location by a retention signal, which in the prokaryotic RPS15a homolog RPS8 is a C-terminal pentapeptide motif ((S/T)-T-(S/T/P)-X-G ) (Tishchenko et al., 2001).

In *E. coli*, RPS8 can bind to both the polycistronic *spc* mRNA (regulating translation of itself and the ten other r-proteins in the *spc* operon) and 16S rRNA through similar structural sites on both RNAs (Cerretti et al., 1988). RPS8 is highly conserved between Bacteria, Archaea and Eukarya with archaeal r-proteins exhibiting the highest degree of

sequence identity with their eukaryotic counter parts (Cerretti et al., 1988; Matheson, 1992; Ramírez et al., 1993). The archaeon *Methanococcus jannaschii* RPS8, which shares 45 – 50% amino acid identity with eukaryotic RPS15a, binds 16S rRNA via a C-terminal S-T-T-Q-G motif. I propose that Arabidopsis type I RPS15a isoforms may be retained in the nucleolus through a similar C-terminal – 18S rRNA interaction.

Originally identified as “housekeeping genes” for their roles in ribosome assembly and function, mutational analysis of r-proteins has identified many as key players in maintaining cellular homeostasis. In Arabidopsis, reduced expression of *RPS5B*, *RPS6B*, *RPS11A*, *RPS13A*, *RPS18A*, *RPS27B*, *RPL3A*, *RPL8A*, *RPL5A/B*, *RPL9C*, *RPL10A*, *RPL10aB*, *RPL19A*, *RPL23C*, *RPL23aA/B*, *RPL24B*, *RPL28A* and *RPL40B* can result in pleiotropic phenotypes including abnormal vascular patterning, aberrant trichome morphology, pointed first leaves, fused leaves, late flowering, retarded root growth, sensitivity to UV irradiation and methyl methane sulphate, prominent leaf serrations, loss of apical dominance, cotyledon defects, reduced fertility and embryo lethality (Revenkova et al., 1999; Ito et al., 2000; Morimoto et al., 2002; Tzafrir et al., 2003; Tzafrir et al., 2004; Nishimura et al., 2005; Degenhardt and Bonham-Smith, 2008; Degenhardt and Bonham-Smith, 2008; Pinon et al., 2008; Fujikura et al., 2009). The majority of these r-protein mutants belong to 2 – 3 member gene families whereas, the *RPS15a* gene family, as previously mentioned, contains five actively transcribed members of two different types. The large number of *RPS15a* family members (potential for compensation upon loss of a single paralog) together with the two types of members adds a layer of complexity to mutational analysis of the *RPS15a* gene family unseen in most other Arabidopsis r-protein gene families.

Here I present data confirming RPS15aA/D and F as cytosolic r-proteins that localize to the nucleolus for SSU assembly and that these type I r-proteins require the terminal seven (or less) C-terminal amino acids for nuclear localization. Using GFP tagged constructs of RPS15aB and E, I confirmed that neither localize to the nucleolus but I was unable to confirm a cellular localization for either other than cytoplasmic. Finally, RNAi analysis suggests that both type I and II families are independently required for plant viability.



## **2.2. Materials and Methods**

### **2.2.1. Plant material and growth conditions**

All plants were grown under a 23°C/17°C temperature regime and 16/8 hour (light/dark) photoperiod of  $\sim 120 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ . Plants grown on soil were potted in sunshine mix number 3 and those grown on plates were sown on  $\frac{1}{2}$  Murashige and Skoog (MS) media (Sigma Aldrich) supplemented with 0.8% phytagar (Invitrogen) and 1.5% sucrose.

For transient expression experiments, tobacco (*Nicotiana tabacum*) cultivar Petit Havana plants were grown under a 23°C/18°C temperature regime and a 16/8 hour (light/dark) photoperiod of  $\sim 170 \mu\text{mol photons m}^{-2} \text{ sec}^{-1}$ . Young leaves from four to six-week-old plants were used for all infiltration experiments.

### **2.2.2. RNAi constructs**

Fragments, 120-200 bp (single KDs) and 350-400 bp (family KDs) in length, were amplified from the 3' untranslated region (UTR: single KDs) and open reading frame (family KDs) for each gene and ligated in sense and antisense orientations into the cloning vector pKANNIBAL (Helliwell and Waterhouse, 2005). The 3' UTR was chosen for single KDs as it exhibited the only sequence of nucleotides that varied substantially between members of the same family; therefore silencing should be specific to the targeted member. The resulting 35S-Hairpin-Terminator cassettes were sub-cloned into the binary vector pART27 and used to transform *Agrobacterium tumefaciens* (Agrobacterium) and subsequently Arabidopsis.

### **2.2.3. Stable transgenics**

Arabidopsis transformations were performed on WT (Col-O) following the protocol outlined by Clough and Bent (1998). Briefly, three-inch pots with 20-30 plants (all with immature flowers) were dipped in a solution of Agrobacterium carrying the RNAi constructs of interest. The Agrobacterium solution was prepared by inoculating 4 mL LB media with -80°C stock of Agrobacterium containing the RNAi construct of interest and grown for 16-18 hours. This culture was used to inoculate 250 mL fresh LB media and allowed to grow overnight. The overnight Agrobacterium culture was centrifuged at 5000

x g for 5 min (at 20°C). The supernatant was removed and approximately 400 mL of 5% (w/v) sucrose containing 0.05% (v/v) Silwet L-77, was added to resuspend the culture to an OD<sub>600</sub> of 0.6 – 0.8. Arabidopsis plants were inverted and dipped into the solution, ensuring that the immature flowers were fully submerged. Excess Agrobacterium solution was allowed to drip off the plants before they were wrapped in plastic wrap and left out of the growth chamber at room temperature overnight. The next day the plastic wrap was removed and plants were returned to the growth chamber until seed set. Following senescence, T<sub>1</sub> seeds were collected, sterilized (70% EtOH wash (8 minutes), 50% bleach wash (8 minutes) and 3 X ultrapure water washes) and plated on ½ MS plates containing 50 µg/mL kanamycin and 100 µg/mL Timentin. Following two weeks of growth, T<sub>1</sub> plants positive for kanamycin selection were transferred to soil to complete their life cycle. Homozygous lines for each RNAi construct were isolated through segregation analysis and used for all subsequent qRT-PCR, root and venation analysis.

#### **2.2.4. Quantitative RT-PCR**

Thirteen-day-old RNAi plants were used for qRT-PCR analysis. Plants were flash frozen in N<sub>2</sub>(l) and stored at -80°C until RNA extraction. RNA was extracted from flash frozen plant tissue from 13-day-old WT, *rps15a/d/f/b* and *e* plants using the Qiagen RNeasy Plant minikit. Approximately 1 µg of total RNA was used to generate oligo dT primed cDNA using the Qiagen Quantitect Reverse Transcription kit. The generated cDNA was used in all subsequent qRT-PCR experiments. A minimum of three biological replicates was completed for each mutant. Primers for each *RPS15a* gene family member (except *RPS15aC*) and *ACT7* (internal control to standardize mutant and WT transcript levels) were designed to produce single amplicons of approximately 200 bps (Appendix C). For each mutant, transcript abundance of all family members (both type I and II) was measured using an iQ5 real-time PCR detection system (BioRad). Amplifications of each gene for each mutant were carried out in triplicate, within the same qRT-PCR run. Data was collected using the iQ5 Optical system software (BioRad) and fold changes in transcript levels were calculated using the  $\Delta\Delta C_t$  method (Dussault and Pouliot, 2006).

### 2.2.5. Fluorescent protein constructs

A heterologous system was used to determine the subcellular localization of each isoform encoded by the *RPS15a* gene family. The coding sequence (CDS: minus the stop codon) of each r-protein was cloned into unique *EcoRI/BamHI* sites in the binary vector pGREENI0029, generating a C-terminal fusion with green fluorescent protein (GFP), all directed by the 35S promoter. CDSs were obtained by RT-PCR (Quantitect reverse transcription kit (Qiagen)) amplification of family member ORFs from total RNA extracted from fifteen-day-old plants using the Qiagen RNeasy plant mini kit. Once cloned into pGREEN all constructs were confirmed by automated sequencing (National Research Council – Plant Biotechnology Institute [NRC/PBI], Saskatoon, SK, Canada) to ensure CDS accuracy and that all fusions were in frame. The pGREENI0029 binary vector was modified by the addition of a tandem repeat of the CaMV 35S promoter (*Apal/EcoRI*), glutathione *S*-transferase (GST) linker (*BamHI/HindIII*), enhanced GFP (EGFP: ClonTech, Palo Alto, CA) (*HindIII/SpeI*) and nopaline synthase (nos) poly(A) signal (terminator) (*SpeI/NotI*) creating pGREENI0029-35S-r-proteinCDS-GST-GFP-nos constructs (Degenhardt and Bonham-Smith, 2008). The addition of GST increased the size of the resulting chimeric protein to that beyond the allowance of the nuclear pore complex (>60 kDa), therefore, ensuring no passive diffusion of r-protein-GFP into the nucleus (Degenhardt and Bonham-Smith, 2008).

Type II members (*RPS15aB/E*) were also cloned in the binary vector pBIN for transient expression in tobacco epidermal cells as fluorescence detection using pGREEN was unsuccessful. GFP-HDEL (endoplasmic reticulum) and STM-RFP (golgi apparatus) cellular markers were obtained from Dr. Frederica Brandizzi (Michigan State University), the pBINmgfp5- $\beta$ -ATPase ( $\beta$ -ATPase) mitochondrial marker was obtained from Dr. David Logan (University of Saskatchewan) and the free mRFP cytoplasmic marker was obtained from Dr. Rory Degenhardt (University of Saskatchewan).

### 2.2.6. *RPS15aA/F* and *D* C-terminal truncations

Full length CDS for *RPS15aD* and *F* were cloned into pBluescript, from which C-terminally truncated fragments were amplified and cloned into pGREEN. From the C-terminal end of *RPS15aD* and *F*, 90, 72, 63 and 21 bp were removed corresponding to  $\Delta 1 - 4$  deletion constructs, respectively.  $\Delta 1$  removed the pentapeptide motif (amino acids 100-

130),  $\Delta 2$  removed three C-terminal amino acids from the pentapeptide motif (amino acids 106-130),  $\Delta 3$  removed all amino acids directly C-terminal to the pentapeptide motif (amino acids 109-130) and  $\Delta 4$  removed the last seven C-terminal amino acids (amino acids 123-130).

#### **2.2.7. Transient expression in tobacco and confocal microscopy**

pGREEN and pSOUP (required to provide pGREEN replication factors: pBIN is a complete binary vector and all subsequent transformation steps were identical to pGREEN/pSOUP) were used together in cotransformation by electroporation of *Agrobacterium* (LBA4404). Colonies positive for the pGREEN constructs were identified using colony PCR with primers specific for each r-protein CDS. Cultures of *Agrobacterium* ( $OD_{600} \sim 0.2 - 0.4$ ) containing pGREEN were injected into the abaxial side of tobacco leaves and the inoculated plants were returned to the growth chamber for 48-72 hours, at which point, leaf sections were viewed with an inverted Zeiss LSM 510 META Confocal Laser Scanning Microscope (CLSM: Jena Germany). Prior to viewing, small sections of infiltrated tobacco leaves were stained with DAPI. Sections were immersed in a 4',6-diamidino-2-phenylindole (DAPI) solution (2  $\mu\text{g}/\text{mL}$ ) and vacuum infiltrated for three hours. For imaging of EGFP-tagged fusion proteins, an Argon laser (488 nm) was used with a 505-530 nm bandpass filter and for imaging of DAPI staining of the nucleic acids a 405 nm diode was used with a 420-460 nm bandpass filter. All images were processed with the Zeiss LSM Image Browser and Picasa image analysis software.

As fluorescence detection was unsuccessful from both pGREEN-RPS15aB/E and pBIN-RPS15aB/E, tobacco leaf sections were coinfiltrated with 5-50  $\mu\text{M}$  of the proteasome inhibitor clasto-lactacystin- $\beta$ -lactone ( $\beta$ -lactone; Sigma-Aldrich). Followed by visualization at 12, 24, 48 and 72 hours after infiltration. Due to a high degree of tissue necrosis and autofluorescence 24 hours post infiltration from necrotic tissue,  $\beta$ -lactone was also injected 24-48 hours post agroinfiltration in an attempt to increase RPS15aB/E-GFP levels to a degree that could be visualized by confocal microscopy and decrease the amount of necrosis. Following  $\beta$ -lactone infiltration, leaf segments were viewed at 12, 24 and 36 hrs.

### **2.2.8. RPS15aE overexpression in *E. coli* and western blots**

RPS15aB and E cDNAs were cloned into the protein expression vector pEHISTEV and the resulting pEHISTEV-RPS15aB/E constructs were used to transform *E. coli* Tuner cells (Novagen, EMD Biosciences, Darmstadt, Germany: Tuner strains are *lacZY* deletion mutants of BL21). Four mL of LB media (containing 50 µg/mL of ampicillin) was inoculated with the pEHISTEV containing Tuner cells and grown overnight (14 – 16 hours). Fresh LB amp media (250 mL) was inoculated with 2 mL of the overnight culture and grown to an OD<sub>600</sub> ~ 0.8, when IPTG was added, to a final concentrations of 0.2 or 0.5 mM, to induced protein expression. One mL samples were taken hourly over six hours, centrifuged at 4°C for 20 min at 4000 x g, washed with ultrapure H<sub>2</sub>O, flash frozen in liquid nitrogen and stored at -80°C. Samples from uninduced cultures were taken alongside samples from induced cultures. Cell culture pellets were boiled in 50-100 uL of 2X SDS loading dye (100 mM Tris-HCl, 200 mM DTT, 4% SDS, 0.2% Bromophenol blue and 20% glycerol) for 15 min and separated by 12% 1D SDS-PAGE (Mini Protean III system – BioRad). Proteins were transferred from the polyacrylamide gel to a 0.45 µM nitrocellulose membrane (BioRad) overnight in a 4°C cooler at 30 V, using a Mini Trans-Blot Cell (BioRad). The membrane was washed with Tris buffered saline (TBS) and blocked with 5% skim milk (in TBS) for 60 min. The membrane was washed 3 – 5 times in TBST (TBS buffer with 10% Tween-20) (5 min/wash) before probing with a 1:1000 dilution of the primary antibody (generated against a RPS15aE peptide: GenScript (Piscataway, New Jersey) generated both the peptide and antibody) for 60 min. The membrane was washed with TBST (5 min) and then probed with a 1:1000 dilution of the secondary antibody (goat anti rabbit IgG conjugated to horseradish peroxidase (HRP): BioRad) for 60 min followed by a final wash in TBST. Protein bands were visualized using the Immunostar HRP substrate kit (BioRad) and a UVP BioImaging Systems EpiChem<sup>3</sup> Darkroom camera with UVP VisionWorks LS software.

### **2.2.9. Genevestigator transcript expression profiling**

Genevestigator ([www.genevestigator.com](http://www.genevestigator.com)) was used (January 2012) to determine developmental stage specific mRNA expression patterns for transcriptionally active *RPS15a*

gene family members. Expression values for each developmental stage were obtained from ATH1: 22K high quality arrays of wild type Arabidopsis (Columbia-O) (Hruz et al., 2008).

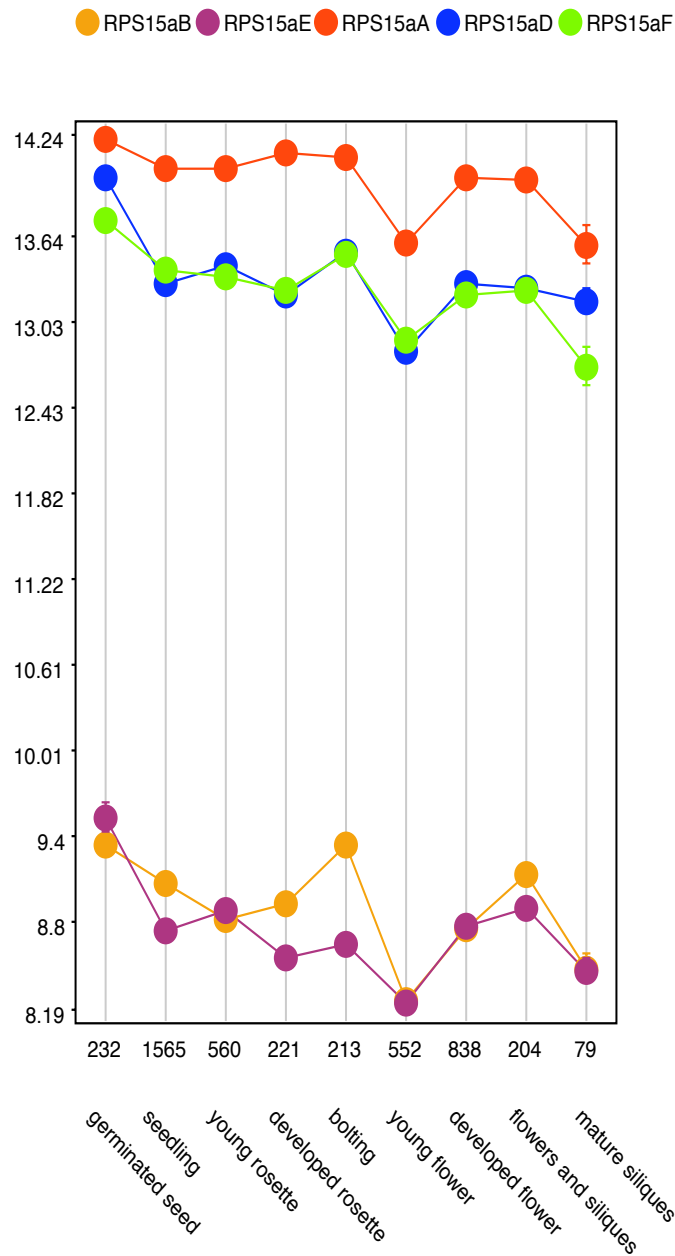
#### **2.2.10. Statistics**

Data was analyzed using a one way ANOVA procedure and MIXED model in SAS 9.2 (SAS Institute. 2008). Assumptions of ANOVA were tested using a Normality test and Levenes test. Means were compared using a Tukey test in SAS 9.2 and the treatments were declared significant at  $P \leq 0.1$ .

### **2.3. Results**

#### **2.3.1. Type I and II *RPS15a* gene family members show similar expression patterns through a variety of developmental stages**

Using Genevestigator, the developmental stage specific mRNA expression patterns for each member of the *RPS15a* gene family was determined (Figure 2.2). The type I paralogs, *RPS15aA/D* and *F*, show similar transcript accumulation and trend throughout all developmental stages (germinated seed through mature siliques) relative to each other. Relative transcript abundance was approximately equal across all developmental stages except young flowers and mature siliques where transcript abundance was lowest. The type II members, *RPS15aB/E*, show a similar trend of expression to type I with respect to developmental stage expression pattern but are accumulated at approximately 65% lower levels relative to type Is.

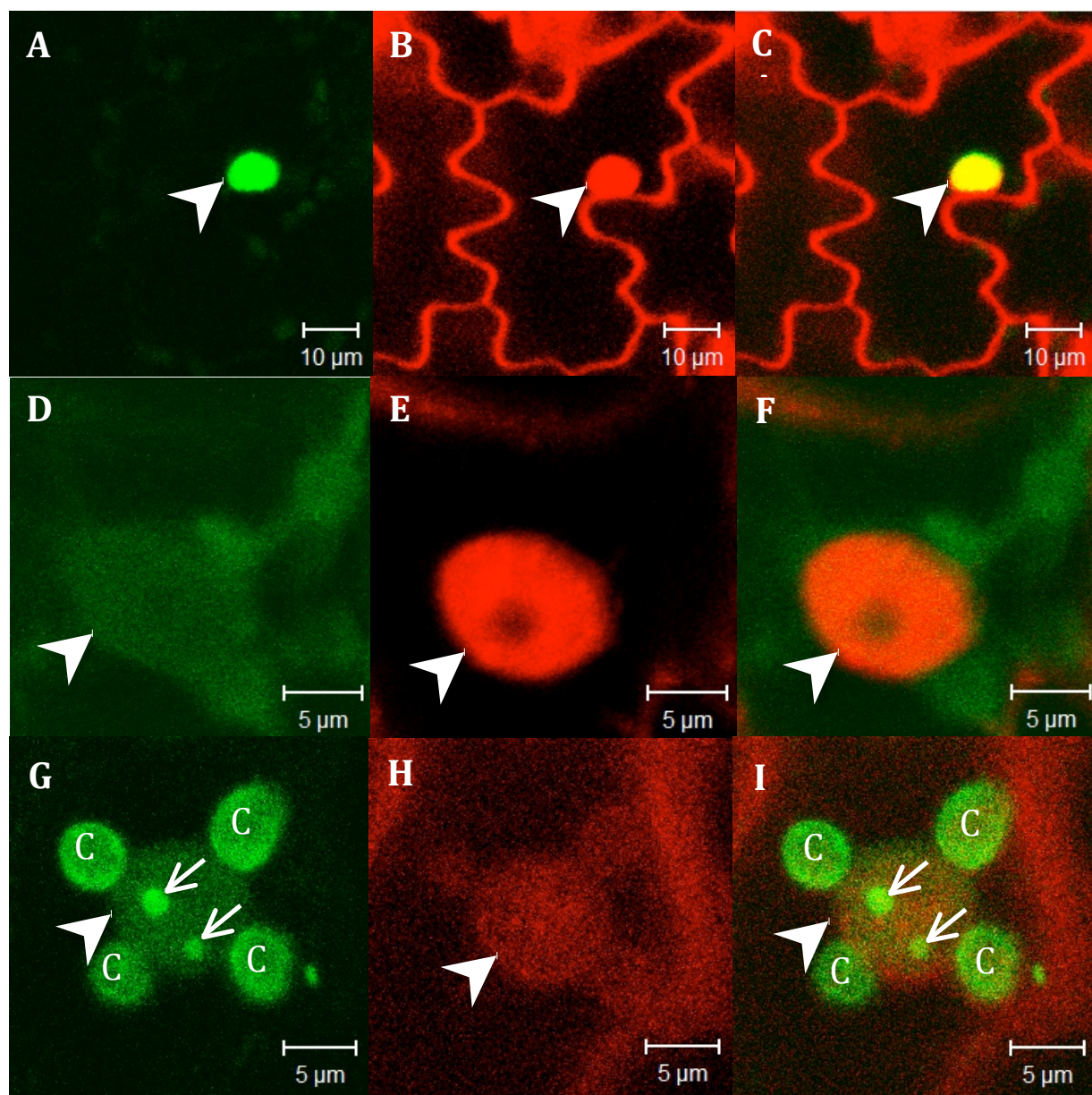


**Figure 2.2. Development stage-specific mRNA expression profiles of each transcriptionally active member of the *RPS15a* family.** Type I members (*RPS15aA/D/F*) are expressed to a higher degree than the type II members (*RPS15aB/E*) although all members show similar expression patterns throughout the developmental stages examined. Y – axis – Level of expression (signal intensity on ATH1: 22K high quality array). X – axis – developmental stage. n = 232 (germinated seedling), n = 1565 (seedling), n = 560 (young rosette), n = 221 (developed rosette), n = 213 (bolting), n = 552 (young flower), n = 838 (developed flower), n = 204 (flowers and siliques) and n = 79 (mature siliques). Analysis carried out in Genevestigator (<https://www.genevestigator.com/gv/>).

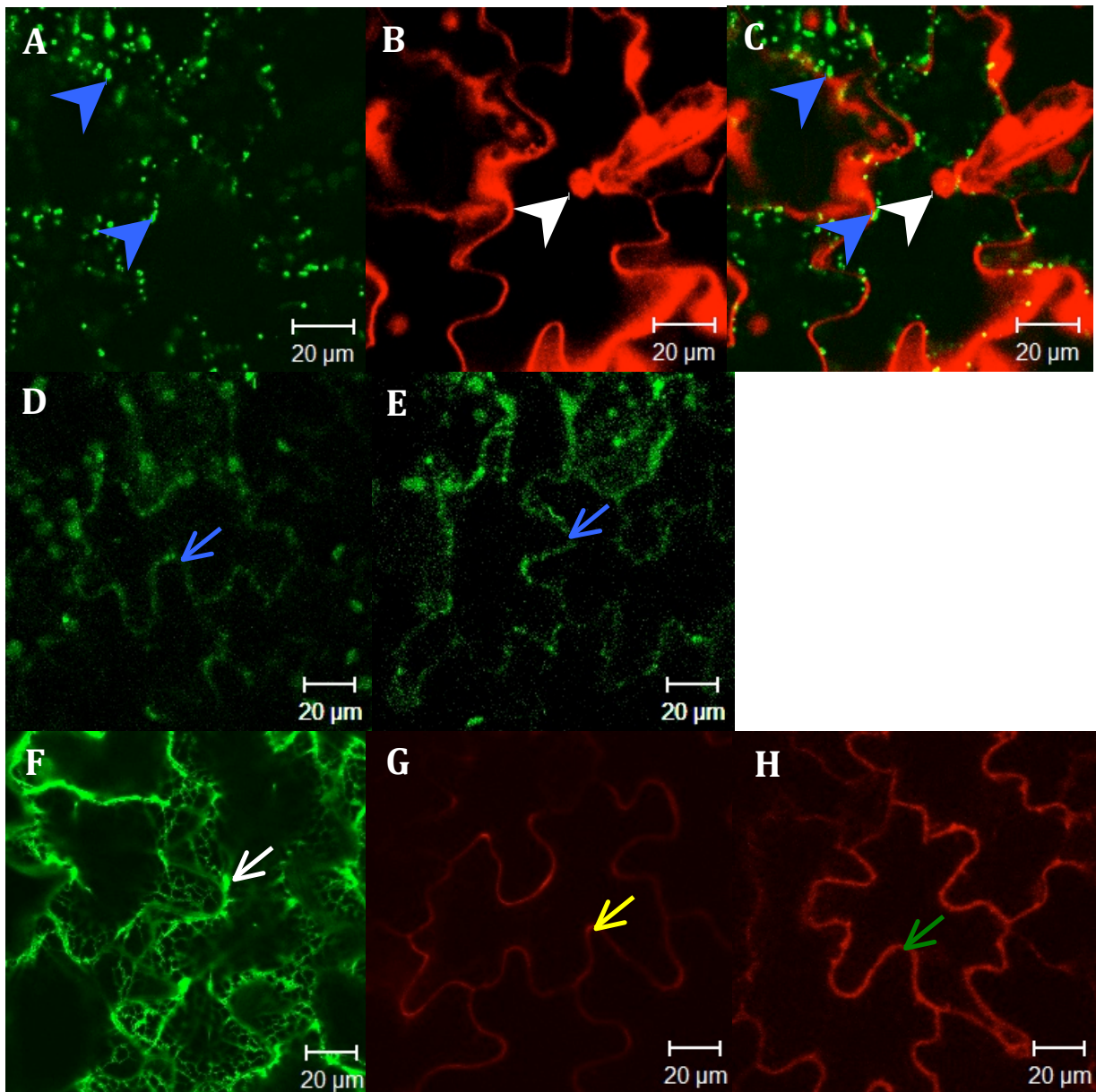
### **2.3.2. Type I RPS15a isoforms exhibit classic r-protein subcellular localization while type II do not**

*RPS15aC* is an inactive pseudogene that does not generate transcript in any tissue under a wide variety of conditions (Hulm et al., 2005), therefore, it has not been included in this study. As previously determined (Hulm et al., 2005) the type I isoforms (RPS15aA/F and D) localize to the nucleus and nucleolus (Figure 2.3 D, G) as would be expected for cytosolic ribosomal proteins; however, questions still surround the type II members, RPS15aB/E. When expressed transiently in tobacco epidermal cells, both proteins, from two different binary vectors (pGREEN and pBIN), failed to show nuclear, nucleolar or mitochondrial localization when compared to the mitochondrial positive control p $\beta$ INBTPaseGFP (Figure 2.4A; but a cytoplasmic signal was observed (Figure 2.4D, E)). Degenhardt (2009; PhD Thesis) determined that in Arabidopsis stable transgenic lines, an excessive production of RPL23aA from the 35S promoter resulted in targeting of the excess r-proteins to the 26S proteasome for degradation. Therefore, in an attempt to visualize RPS15aB/E isoforms in tobacco, pGREEN and pBIN constructs were co-infiltrated with the proteasome inhibitor  $\beta$ -lactone. Using a variety of concentrations (5-50  $\mu$ M), infiltration OD<sub>600</sub> (0.2-0.6) and time frames (24-72 hours post-infiltration) no mitochondrial localization was observed (data not shown). RPS15aB/E localization was further compared to that of a variety of cellular markers including the nucleus/nucleolus (FIBRILLARIN-GFP), endoplasmic reticulum (ER: HDEL-GFP), golgi apparatus (ST-mRFP) and cytoplasm (Free mRFP) (Figure 2.4F, G and H) and found to be most similar to that for the cytoplasmic marker (Figure 2.4H). These data support the suggestion by Chang et al. (2005) that RPS15aB/E may be recruited to cytoplasmic ribosomes following small subunit export from the nucleus to the cytoplasm. However, the cytoplasmic signal may also be a result of strong 35S promoter driven expression of the chimeric proteins.





**Figure 2.3. Type I RPS15a cellular localization.** (A, D, G) GFP images, (B,E,H) DAPI images and (C,F,I) merged images. (A-C) Fibrillarin nuclear localization. (D-F) RPS15aD localizing to the nucleus. (G-I) RPS15aA/F exhibiting nuclear/nucleolar localization. White arrows indicate nucleoli, white arrowheads indicate nuclei. C – chloroplasts.



**Figure 2.4. Type II RPS15a cellular localization along with several cellular markers.** (A,B,C) Positive control for mitochondrial localization, (A)  $\beta$ -ATPase, (B) DAPI, (C) merged image. (D) RPS15aB exhibiting weak cytoplasmic localization (detector gain had to be increased to illustrate the weak signal). (E) RPS15aE displaying a weak cytoplasmic signal as in D. (F) GFP-HDEL, positive control for the ER. (G) ST-mRFP, positive control for golgi. (H) Free mRFP, positive control for the cytoplasm. White arrow heads indicate nuclei, light blue arrows indicate weak RPS15aB/E cytoplasmic localization, blue arrowheads indicate mitochondria, white arrow indicates ER, yellow arrow indicates golgi and green arrow indicates cytoplasm.

### **2.3.3. Nuclear localization of the type I RPS15a isoforms requires the C-terminal seven amino acids of the protein**

A consensus pentapeptide motif ((S/T)-T-(S/T/P)-X-G) located in the C-terminal region of the RPS15a prokaryotic homolog RPS8 is required for binding 16S rRNA (Figure 2.5). A similar sequence is present in both type I (TTSAG) and type II (TTPDG) RPS15a proteins. A series of C-terminal truncations of the RPS15aA/F (RPS15aA and F share 100% amino acid identity) and D isoforms were made to investigate the role of this sequence in nuclear/nucleolar localization/retention. Four truncations (**Δ1-Δ4**) of the two cytoplasmic isoforms were designed to keep or remove the pentapeptide motif (Figure 2.6A).

The **Δ1** and **Δ2** chimeric proteins, lacking the pentapeptide motif, showed no nuclear localization when expressed in tobacco epidermal cells. GFP localization was also not detected for either the **Δ3** or **Δ4** constructs suggesting that r-protein localization to the nucleolus is dependent on the C-terminal seven amino acids of the protein (Figure 2.6B).

RPS15aA	MVRISVLNDALKSMYNAEK <b>RGK</b> QVMIRPSSKVIKFLIVMQKHGYIGEFYVDDHRSGK	60
RPS15aD	MVRISVLNDGLKSMYNAEK <b>RGK</b> QVMIRPSSKVIKFLIVMQKHGYIGEFYVDDHRSGK	60
RPS15aF	MVRISVLNDALKSMYNAEK <b>RGK</b> QVMIRPSSKVIKFLIVMQKHGYIGEFYVDDHRSGK	60
RPS15aB	MGR-RILNDALRTIVNAEK <b>RGK</b> ASVELKPVSTMSSFLKIMKEKGYIKNFQVHDPHRVGR	59
RPS15aE	MGR-RILNDALRTIVNAEK <b>RGK</b> ASVELKPISTMSSFLRIMKEKGYIKNFQVYDHRVGR	59
<i>H.sapiens</i>	MVRMNVLADALKSINNAEK <b>RGK</b> QVLIRPSSKVIKFLIVMQKHGYIGEFYVDDHRSGK	60
<i>S.cerevisiae</i>	MTRSSVLADALNAINNAEK <b>RGK</b> QVLIRPSSKVIKFLQVMQKHGYIGEFYVDDHRSGK	60
<i>R.norvegicus</i>	MVRMNVLADALKSINNAEK <b>RGK</b> QVLIRPSSKVIKFLIVMQKHGYIGEFYVDDHRSGK	60
<i>M.jannaschii</i>	MSLMDPLANALNHISNCERVGKKVVIKPAKSLIGRVLKVMQDNGYIGEFYVDDHRSGK	60
<i>T.thermophilus</i>	MLT-DPIADMLTRIRNATRVYKESTDV-PASRFKEEILRILAREGFYKGYERVDVDGKPY	58
<i>E.coli</i>	MSMQDPIDMLTRIRNGQAANKAAVTM-PSSKLKVAIANVLKEEGFIEDFK-VEGDTKPE	58
RPS15aA	IVVELNGRLNKCGLVISPRFDVGVEIEGWTA-----RLLPS--RQFGYIVL <b>TTSAG</b>	109
RPS15aD	IVVELNGRLNKCGLVISPRFDVGVEIEGWTA-----RLLPS--RQFGYIVL <b>TTSAG</b>	109
RPS15aF	IVVELNGRLNKCGLVISPRFDVGVEIEGWTA-----RLLPS--RQFGYIVL <b>TTSAG</b>	109
RPS15aB	ITVDLQGRVNDCKALTYRQDVKANEIGQYTE-----RTLPT--RQWGYIVI <b>TTDPG</b>	108
RPS15aE	ITVDLQGRVNDCKALTYRQDVRAKEIEKYTE-----RTLPT--RQWGYVVI <b>TTDPG</b>	108
<i>H.sapiens</i>	IVVNLTGRLNKCGLVISPRFDVQLKDLEKWQN-----NLLPS--RQFGYIVL <b>TTSAG</b>	109
<i>S.cerevisiae</i>	IVVQLNGRLNKCGLVISPRFNVKIGDIEKWTA-----NLLPA--RQFGYIVL <b>TTSAG</b>	109
<i>R.norvegicus</i>	IVVNLTGRLNKCGLVISPRFDVQLKDLEKWQN-----NLLPS--RQFGYIVL <b>TTSAG</b>	109
<i>M.jannaschii</i>	FKVELIGKINKCGAIKPRFPVKFGYEFKFEK-----RYLPA--RDFGILIV <b>STTQG</b>	109
<i>T.thermophilus</i>	LRVYLKYGPRRQGP-DPRPEQVIHHIRRIKPGRRVYVGVKEIPRVRRGLGIAIL <b>STSKG</b>	117
<i>E.coli</i>	LELTLK-YFQGKAVVESIQRVSRPGLRIYKR---K-----DELPKVMAGLGIAV <b>STSKG</b>	109
RPS15aA	IMDHEEARRKNVGG <b>KVLGFFY</b>	130
RPS15aD	IMDHEEARRKNVGG <b>KVLGFFY</b>	130
RPS15aF	IMDHEEARRKNVGG <b>KVLGFFY</b>	130
RPS15aB	ILDHEEAIKRNVGGQVLGFFH	129
RPS15aE	ILDHEEAIKRNVGGQVLGFFY	129
<i>H.sapiens</i>	IMDHEEARRKHTGGKILGFFF	130
<i>S.cerevisiae</i>	IMDHEEARRKHVSGKILGFVY	130
<i>R.norvegicus</i>	IMDHEEARRKHTGGKILGFFF	130
<i>M.jannaschii</i>	VMSHEEAKKRGLGGRLLAYVY	130
<i>T.thermophilus</i>	VLTDRARKLGVGGELICEVW	138
<i>E.coli</i>	VMTDRAARQAGLGGEIICYVA	130

**Figure 2.5. Nuclear and nucleolar localization sequences in prokaryotic RPS8 and eukaryotic RPS15a.** Yellow sequence – pentapeptide motif (in prokaryotes this motif interacts with both mRNA and rRNA); black box – putative ‘RGK’ nucleolar localization signal in yeast; turquoise sequence – experimental nuclear localization signal in yeast; red sequence – seven amino acids experimentally determined to be essential for nuclear localization in Arabidopsis.

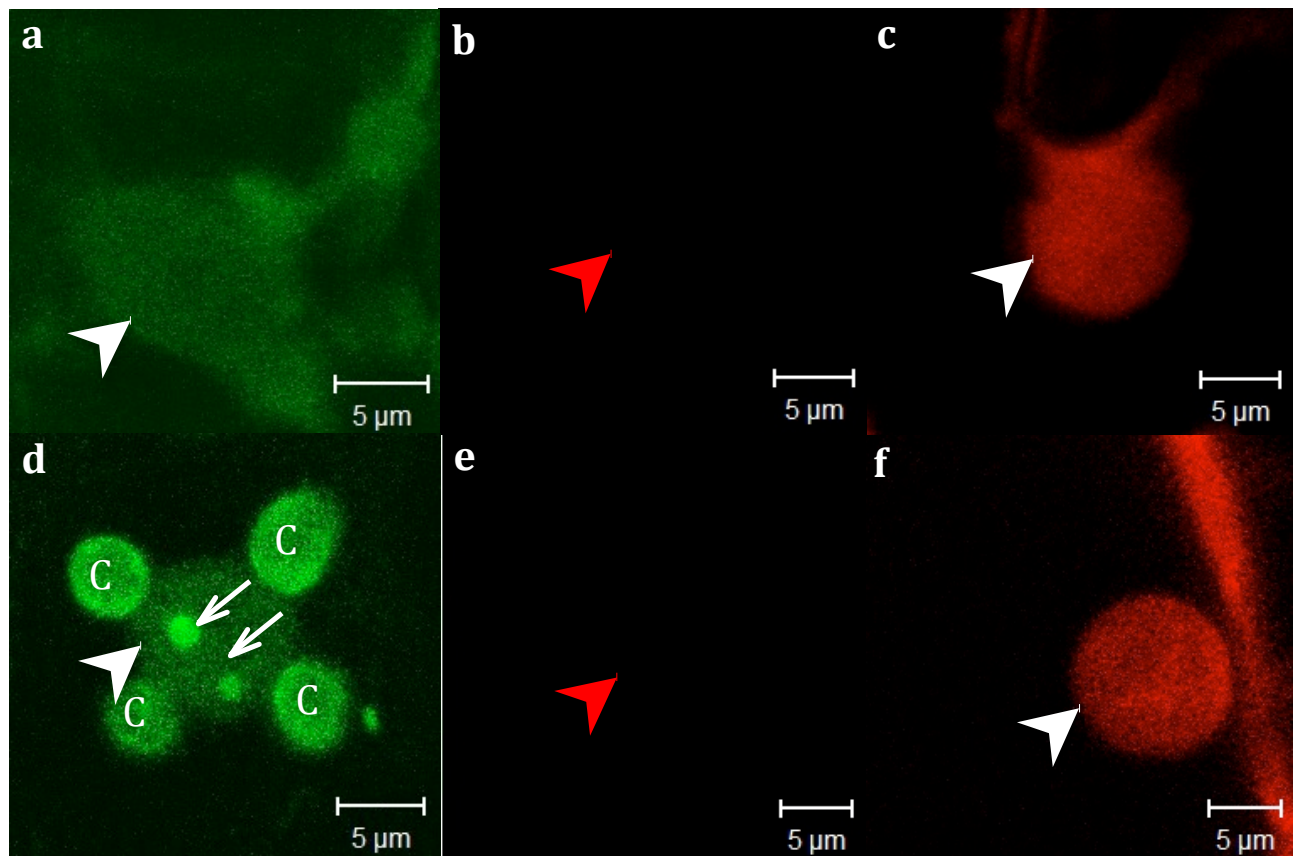


**Figure 2.6. RPS15aA/F and D C-terminal truncations.** (A) *RPS15aA/F* encode identical proteins.  $\Delta 1$  deletion removed the pentapeptide motif completely,  $\Delta 2$  removed 'SAG' of the motif,  $\Delta 3$  removed all amino acids 3' to the motif and  $\Delta 4$  removed the last seven C-terminal amino acids. (B) (a) Full length RPS15aD nuclear localization. (b) All RPS15aD C-terminal deletions disrupted nuclear localization (Figure is representative of all deletion constructs). (c) DAPI staining of the nucleus in b. (d) Full length RPS15aA/F nuclear and nucleolar localization. (e) All RPS15aA/F C-terminal deletions disrupted nuclear localization (Figure is representative of all deletion constructs). (f) DAPI staining of the nucleus in e. White arrow heads – nucleus, white arrows – nucleolus, red arrow heads – disrupted nuclear/nucleolar localization, C – chloroplasts.

**A**

<b>RPS15aD</b>	
<b>Full Length</b>	
MVRISVLNDGLKSMYNAEKRGRQVMIRPSSKVIKFLIVMQKHGYIGEFYVDDHRS	
GKIVVELNGRLNKCGLVISPRFDVGVKEIEGWTARLLPSRQFGFIVLTTSAGIMDHEEA	
RRKNVGGKVLGFFY	
$\Delta 4$	$\Delta 1 \quad \Delta 2 \quad \Delta 3$
<b>RPS15aA/F</b>	
<b>Full length</b>	
MVRISVLNDALKSMYNAEKRGRQVMIRPSSKVIKFLIVMQKHGYIGEFYVDDHRS	
GKIVVELNGRLNKCGLVISPRFDVGVKEIEGWTARLLPSRQFGYIVLTTSAGIMDHEEA	
RRKNVGGKVLGFFY	
$\Delta 4$	$\Delta 1 \quad \Delta 2 \quad \Delta 3$

**B**



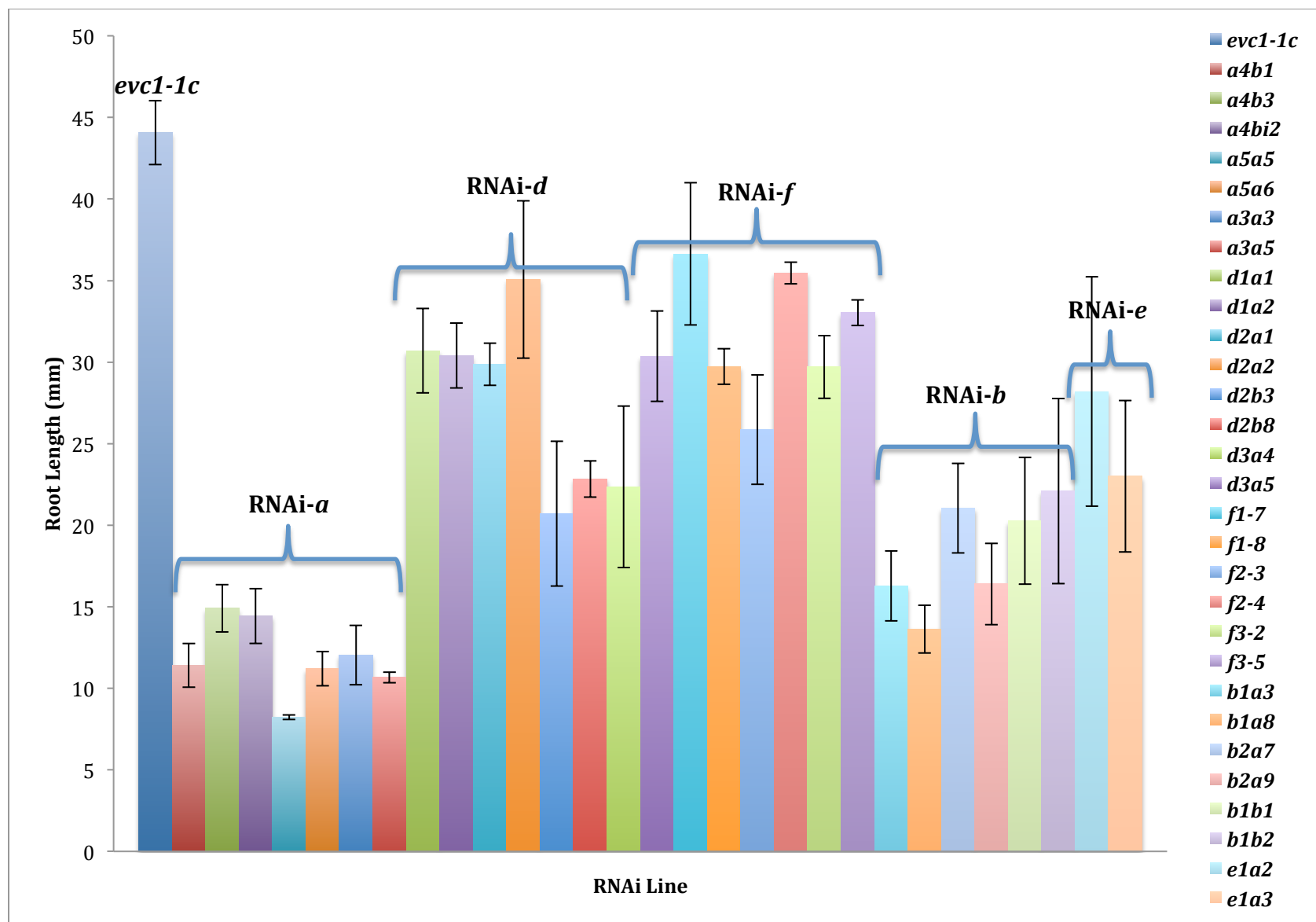


#### 2.3.4. Delayed root development in individual type I and II RNAi lines

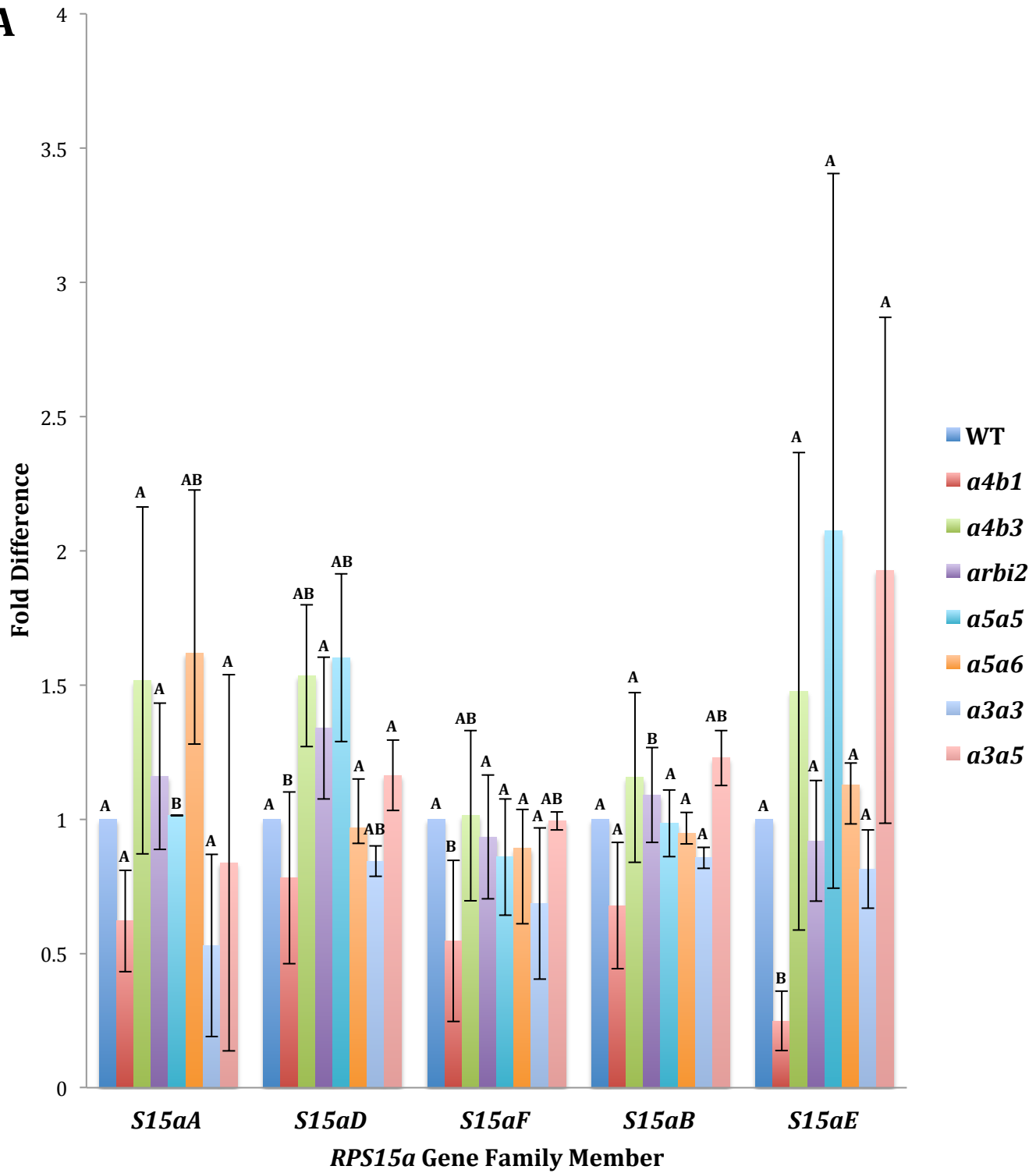
To determine the need for type I and II *RPS15a* members in normal plant development, RNAi constructs for each member were generated. KDs of both type I and type II families proved to be lethal with no transgenic lines obtained for either family (three *Arabidopsis* transformation experiments). This outcome confirms the requirement of at least one member of each family for normal plant growth and development. All individual gene RNAi lines showed decreased root growth compared to the empty vector control (*evc1-1c*: 44.1 mm) with RNAi-*a* lines showing the shortest roots (8.2 → 14.9 mm) followed by the RNAi-*b* lines (13.6 → 22.1 mm). RNAi-*d* and -*e* lines showed the least deviation from *evc1-1c* with root lengths of 20.7 → 35.1 mm and 23 → 28.2 mm, respectively (Figure 2.7). Root morphology was not affected. Given adequate time (RNAi-*d* and -*f*: 1 week behind *evc1-1c*, RNAi-*b* and -*e*: 1-2 weeks behind *evc1-1c* and RNAi-*a*: 2-3 weeks behind *evc1-1c*), all RNAi lines matured and viable seed was produced.

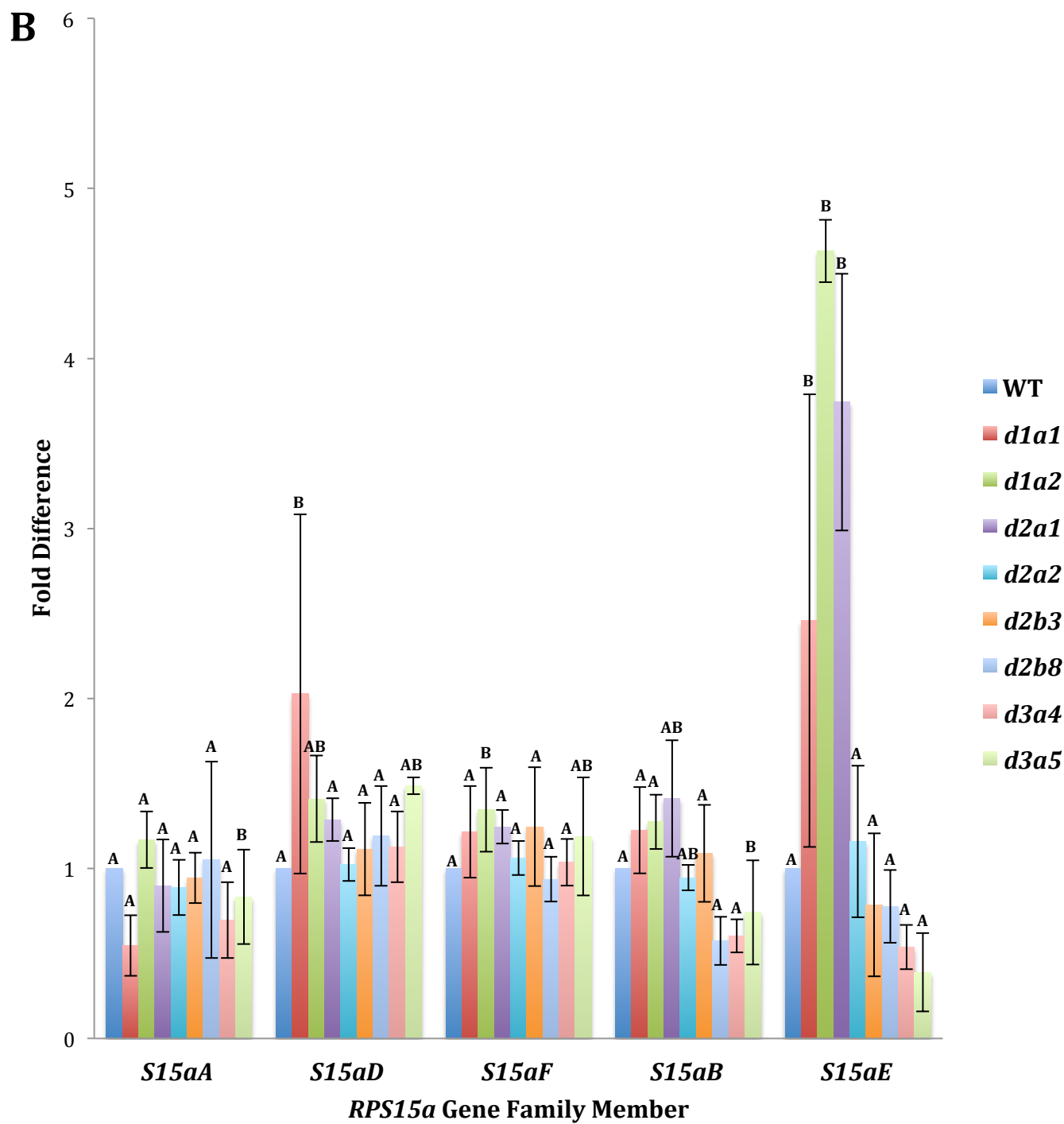
KDs, of each individual gene, were less conclusive as few single RNAi lines showed consistent levels of reduced transcript (Figure 2.8A, B, C). While two RNAi-*a* lines (*a4b1* and *a3a3*) showed decreased transcript levels (1.6 and 1.8 fold, respectively) overall, there was no correlation between transcript level and root length (Figure 2.7), even though all RNAi-*a* lines showed a 75% decrease in root length compared to wildtype (*evc1-1c*). None of the RNAi-*d* lines showed a significantly reduced transcript level but like the RNAi-*a* lines, all RNAi-*d* lines showed a 33% decrease in root length compared to *evc1-1c*. Five of the six RNAi-*f* lines showed reduced *RPS15aF* transcript level (*f1-7* – 4 fold, *f1-8* – 1.6 fold, *f2-3* – 3.2 fold, *f2-4* – 1.4 fold and *f3-5* – 1.6 fold) however the reduction in root length was equivalent (~33%) to that of the RNAi-*d* lines (Figure 2.7). *a4b1* and *a3a3* show decreased transcript levels in all five genes but this global decrease in *RPS15a* genes was not reflected in a more substantial decrease in root length. *e1a3* showed a slight decrease in *RPS15aB* transcript levels while *b2a9* and *b1b1* showed small decreases in *RPS15aE* transcript levels, but no decrease in transcript abundance of the targeted gene was detected. A 45% and 40% decrease in root length was evident in RNAi-*b* and -*e* lines, respectively (Figure 2.7).

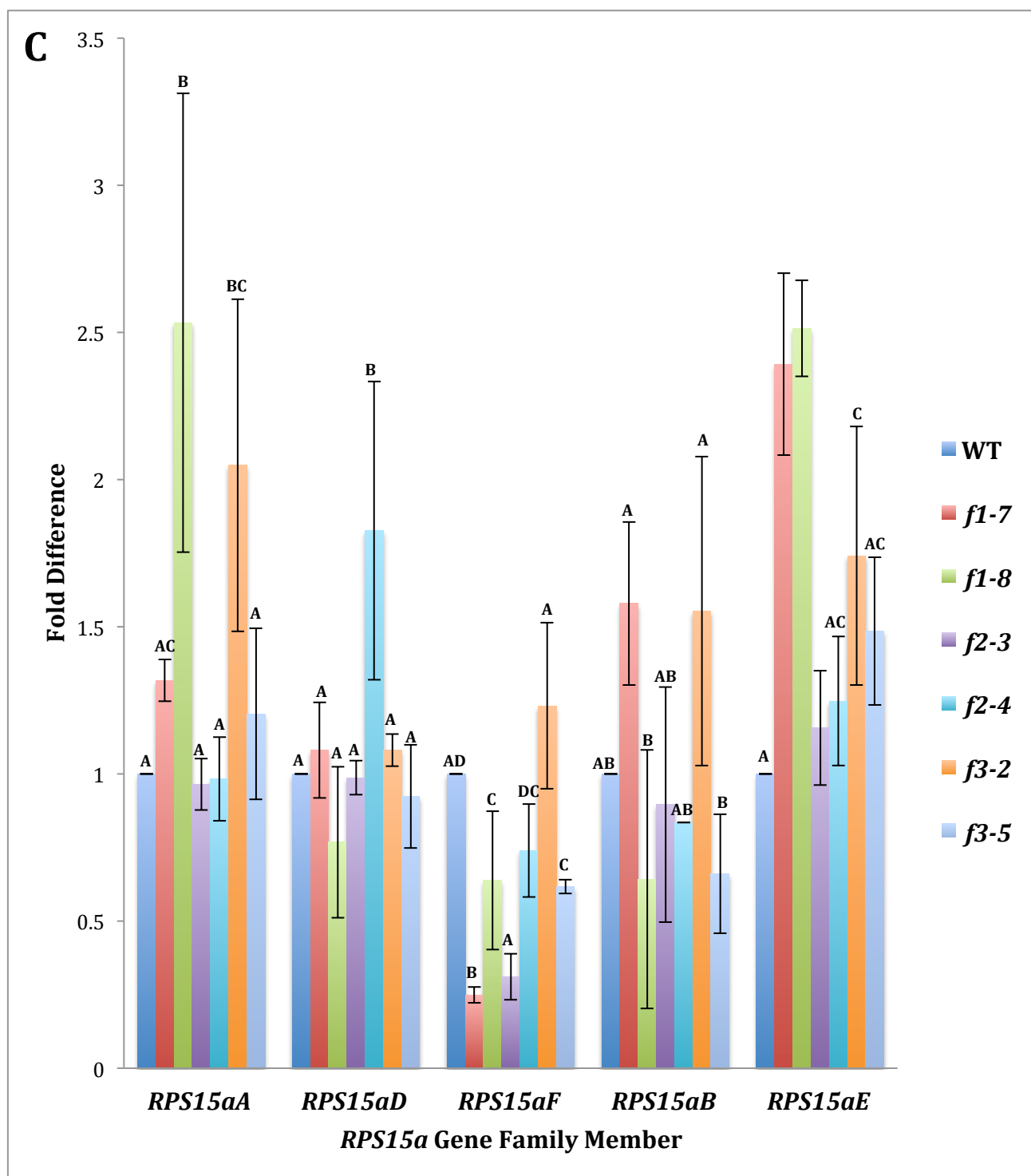
None of the abnormal venation patterns common in many r-protein mutants were identified in the 1<sup>st</sup> to 4<sup>th</sup> leaves of any of the type I or type II individual RNAi lines.

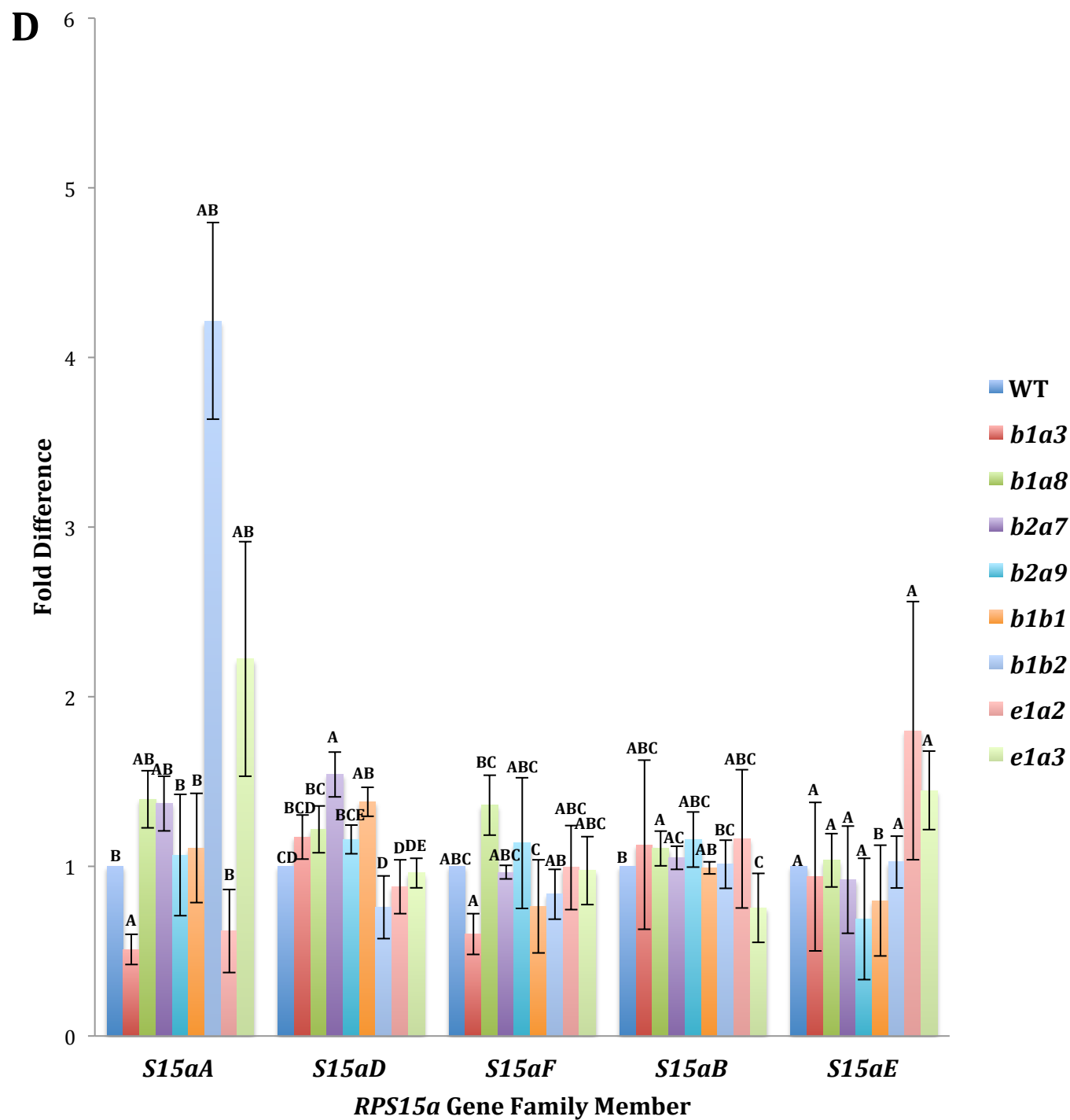


**Figure 2.7. Root length of individual 19-day-old *RPS15a* RNAi lines.** Root length of all individual type I and type II RNAi lines. All RNAi lines showed decreased root growth compared to the *evc1-1c* with the RNAi-*a* lines showing the greatest reduction. n = 3 (3 biological replicates, 10 plants within each biological replicate were measured for each RNAi line).

**A**







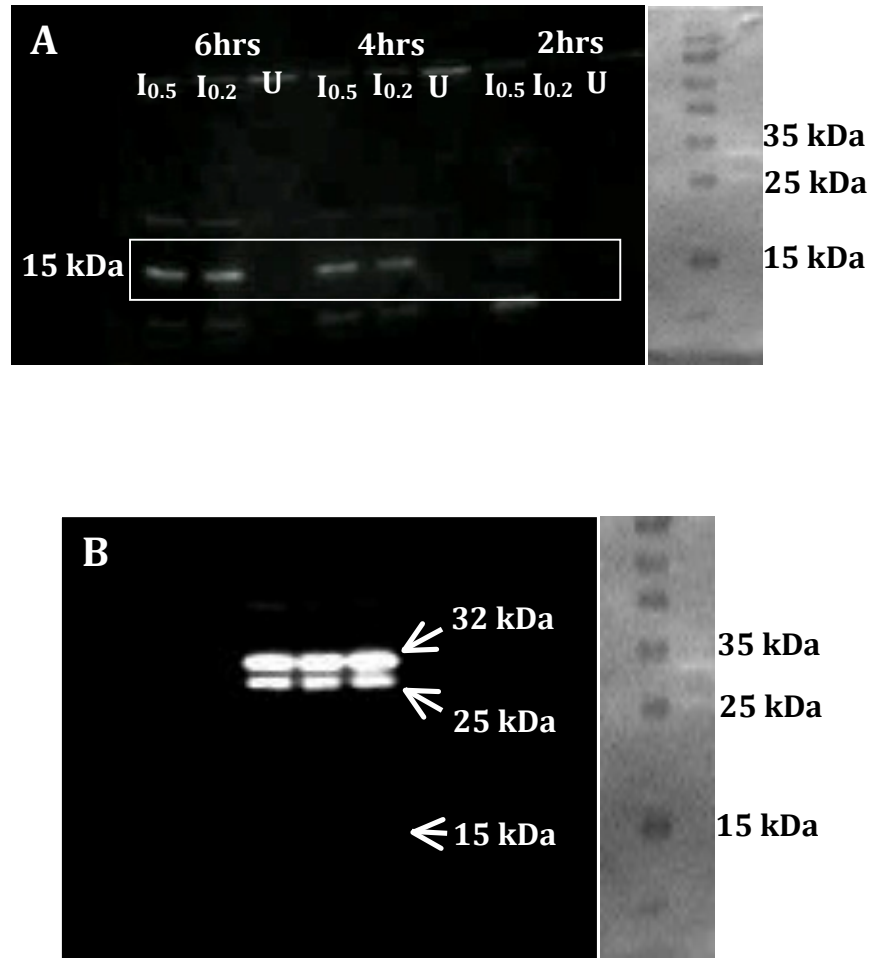
**Figure 2.8. qRT-PCR of individual *RPS15a* RNAi lines** (A) RNAi-*a* lines qRT-PCR. (B) RNAi-*d* lines qRT-PCR. (C) RNAi-*f* lines qRT-PCR. (D) RNAi-*b* and -*e* lines qRT-PCR. Transcript abundance of each active *RPS15a* family member was measured in each of the RNAi lines. A Tukey test was carried out between each of the plant lines. Means of 3 biological replicates per line ( $\pm$  standard error) with same letters do not differ significantly ( $P \leq 0.1$ ). Three technical replicates were performed for each RNA sample. Analysis is only between mutants for each *RPS15a* gene.



### **2.3.5. RPS15aE is not detected in total protein from Arabidopsis seedlings**

An anti-peptide antibody specific to RPS15aB/E, but not type I RPS15a isoforms, was generated (anti-RPS15aE). The peptide predicted to give the highest level of antigenicity was the fourteen amino acid stretch 84-'EIEKYTERTLPTRQ'-97 that contained only two mismatches between RPS15aB and E and was only 57% similar to RPS15aA/F and D. Expressed RPS15aB/E in *E. coli* Tuner cells after induction with either 0.2 or 0.5 mM IPTG, confirmed the functionality of the antibody (Figure 2.9A).

Using this antibody, no 15 kDa (predicted size of RPS15aB and E) proteins were identified in Arabidopsis total protein extracts from 15 day old plants but two strong bands of protein at 25 and ~32 kDa reacted with the antibody (Figure 2.9B). A similar sample of total protein from 15 day old Arabidopsis plants was separated by 1D SDS-PAGE, stained with Coomassie Brilliant Blue R-250 and the stained 15, 25 and 32 kDa bands excised and sent to the UVic Proteomics Center (Victoria, BC) for LC-MS/MS sequencing. RPS15aB/E were not identified in any of the gel fragments suggesting that these proteins were not present at levels detectable by LC-MS/MS and that the antibody reacted with other proteins.



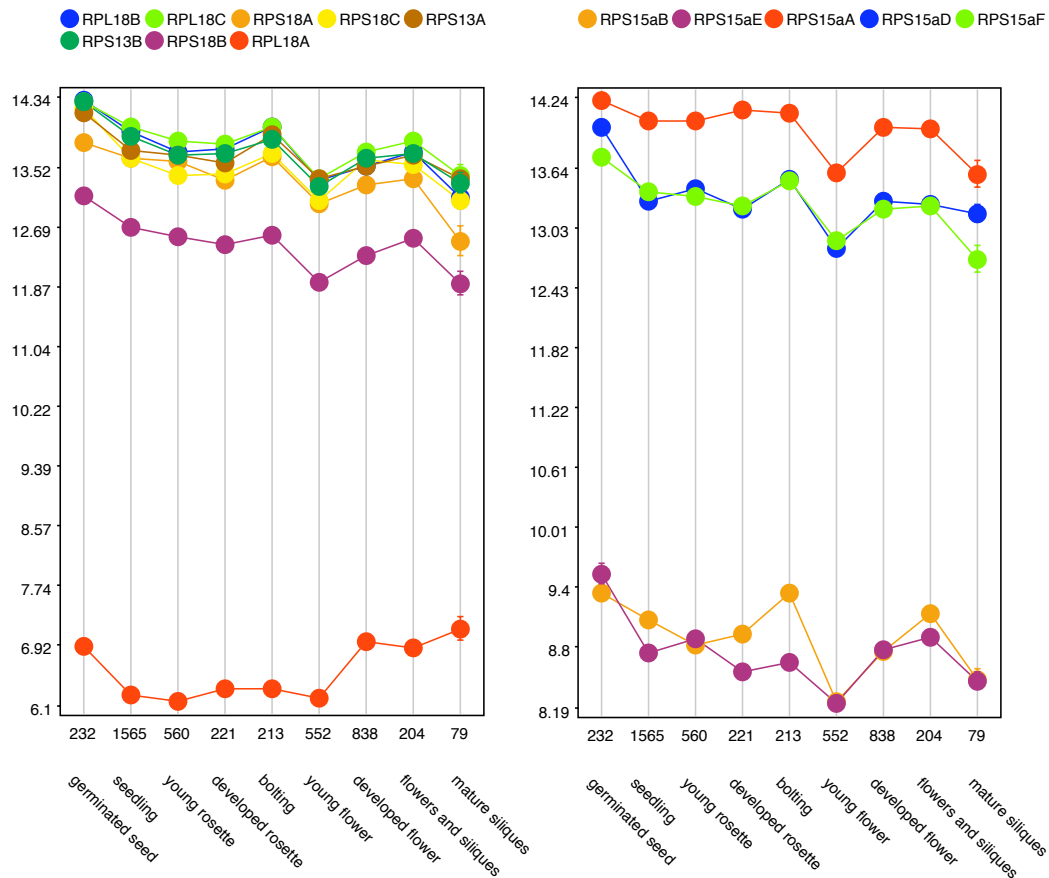
**Figure 2.9. Western blot of total protein from 15-day-old Arabidopsis plants probed with anti-RPS15aE antibody.** (A) RPS15aE was overexpressed in *E. coli* Tuner cells. The predicted molecular mass of RPS15aE and B is 15 kDa, corresponding to the band present in the induced samples at four and six hours post induction with IPTG. (B) Total Arabidopsis protein probed with the anti-RPS15aE antibody. Strong signals were produced at 32 and 25 kDa, but not 15 kDa. The 32, 25 and 15 kDa bands were excised from 1D-PAGE gels and sequenced by LC-MS/MS. RPS15aB and E were not identified in any band.

## 2.4. Discussion

Most Arabidopsis r-protein gene families contain multiple cytoplasmic members that upon translation are transported to the nucleolus for SSU and LSU assembly. Here, I have shown that the *RPS15a* gene family contains two members (*RPS15aB* and *E*) that are not transported to the nucleolus and the function of which remains unknown. Type I members all localized to the nucleus and nucleolus (along with cytoplasm) consistent with that found for many Arabidopsis small and large subunit r-proteins (*RPS13A/B*, *RPS18A/B/C*, *RPL18B/C* and *RPL23aA/B*) (Degenhardt and Bonham-Smith, 2008). A RNAi mediated KD of all three transcriptionally active type I family members was lethal, similar results have been shown in many r-protein families where a KD of a dominant paralog of a family resulted in plant lethality (*RPS5*, *RPS6*, *RPS11*, *RPL3*, *RPL8*, *RPL19*, *RPL23C*, *RPL23a* and *RPL40*) (Weijers et al., 2001; Morimoto et al., 2002; Tzafrir et al., 2003; Tzafrir et al., 2004; Degenhardt and Bonham-Smith, 2008).

In contrast to *RPS13*, *RPS18* and *RPL18* (Chapter 4) where expression of the GFP-tagged proteins in tobacco epidermal cells occurred in between 25-50% of cells, expression of *RPS15aA/F* and *D* was remarkably low. Nuclear/nucleolar localization was detected in only approximately 0.5 – 2% of viewed cells, possibly reflecting the cellular requirements of each individual r-protein. Interestingly, transcript expression profiles for each member of the four gene families (*RPS15a*, *RPS18*, *RPS13* and *RPL18*) are remarkably similar (except for type II *RPS15a*'s and *RPL18A* – the latter being a putative pseudogene) from germinating seedling to mature silques, suggesting some level of translational or posttranslational regulation could be responsible for the low levels of protein expression in the transient expression experiments (Figure 2.10).

In mammalian cells, a dual approach of MS analysis and fluorescence microscopy has successfully shown the import and export of a variety of nucleolar proteins, including r-proteins (Lam et al., 2007). It was found that cytoplasmic r-proteins are both the most abundantly produced nucleolar proteins and accumulate in the nucleolus to a higher degree than any other nucleolar protein. To ensure that the r-protein amount is not rate limiting in ribosome biogenesis, r-protein import into the nucleolus exceeds that required



**Figure 2.10. Development stage-specific transcript expression profiles of RPS13, RPS15a, RPS18 and RPL18 gene families.** All family members (except for type II *RPS15a* members and RPL18A) are expressed to approximately the same level across all developmental stages. Y – axis – Level of expression (signal intensity on ATH1: 22K high quality array). X – axis – developmental stage. n = 232 (germinated seedling), n = 1565 (seedling), n = 560 (young rosette), n = 221 (developed rosette), n = 213 (bolting), n = 552 (young flower), n = 838 (developed flower), n = 204 (flowers and siliques) and n = 79 (mature siliques). Analysis carried out in Genevestigator (<https://www.genevestigator.com/gv/>)

for SSU and LSU assembly, with excess unincorporated r-proteins degraded in the nucleoplasm (Lam et al., 2007). However, not all r-proteins accumulate in the nucleolus at the same rate. The same study compared accumulation rates of RPL27 and RPL5 and it was found that RPL5 accumulated in the nucleolus at a much slower rate than RPL27. The decreased rate was suggested to be due to the interaction between RPL5 and 5S rRNA in the nucleoplasm (possibly due to its role as a positive regulator of p53 activation (Steitz et al., 1988; Horn and Vousden, 2008)) as cytoplasmic levels of RPL5 remained consistent with that of other r-proteins (Lam et al., 2007). Degradation of excess r-protein was mediated by the 26S proteasome (Matsumoto et al., 2005; Lam et al., 2007). A similar mechanism has recently been described in plants where overexpressed isoforms of RPL23a were stabilized in the presence of proteasome inhibitors and were found to be polyubiquitinated (Degenhardt, 2009). In HeLa cells, treatment with the proteasome inhibitor MG132 increased the concentration of nucleolar r-proteins (Andersen et al., 2005), further implicating rapid r-protein turnover mediated by the 26S proteasome. These data suggest a similar mechanism may be responsible for the weak expression of the RPS15a isoforms in tobacco epidermal cells when compared to that for the RPS13, RPS18 and RPL18 isoforms (Chapter 4). RPS15a isoforms may be interacting with an as yet unidentified protein complex (or rRNA) therefore slowing or inhibiting its transport into the nucleolus or r-protein turnover may be exceptionally high for type I members encoded by the *RPS15a* gene family making GFP accumulation extremely low.

Due to the ambiguity surrounding the functions(s) of RPS15aB and E in the cell, cellular localization of both isoforms was investigated. With two different binary vectors (pGREEN and pBIN), RPS15aB and E expression was only detected at low levels in the cytoplasm. Originally believed to be nuclear encoded mitochondrial proteins, I compared expression of RPS15aB and E to that of pBIN $\beta$ ATPase (a positive control for mitochondrial localization (Logan and Leaver, 2000)). The small  $\sim 1.0 \mu\text{m}$  punctate structures evident in tobacco cells expressing pBIN $\beta$ ATPase were not seen in cells expressing RPS15aB and E. Three things may be happening; (1) large quantities of RPS15aB and E are not required in the cell, such that the genes and transgenes are being transcribed but the resulting transcripts or proteins are rapidly degraded, (2) the copy number of RPS15aB and E in

mitochondrial ribosomes is insufficient to visualize via a GFP tag and confocal microscopy or (3) the N-terminal GFP tag may be blocking mitochondrial transport. My research does not support the second possibility as the cytoplasmic signal obtained for RPS15aB and E expression was less than that observed for any other Arabidopsis r-protein investigated in the Bonham-Smith lab to date (RPL23aA/B, RPS15aA/D/F, RPS13A/B, RPS18A/B/C, RPL18B/C, RPS3aA/B, RPS8A/B, RPL7aA/B and RPL15A/B). Even the overall weak signals obtained for RPS15aA/F and D were more pronounced in the cytoplasm than RPS15aB and E expression, suggesting that excess RPS15aB and E generated from the 35S promoter, was rapidly degraded, most likely by the 26S proteasome. While it appears that only small amounts of both/either RPS15aB and E are required in the plant cell, one or both are absolutely required for normal plant growth and development as transgenic RNAi mediated KD lines targeting both type II members were unable to be generated. With no transgenics obtained for the type II family KD, I can conclude that some threshold of a type II member(s) is required during early seed or plant development.

The non-mitochondrial but cytoplasmic localization of RPS15aB and E supports the possibility that both may be recruited to cytoplasmic ribosomes (or SSU) following subunit export from the nucleolus to the cytoplasm. This is indeed the case with several phosphorylated r-proteins that comprise the acidic lateral stalk on the 60S subunit (Wool et al., 1991). In plants, the lateral stalk is comprised of two copies each of P1 and P2 r-proteins and single copies of P0 and RPL12 (L7/L12, L10 and L11, respectively in prokaryotes) (Gonzalo and Reboud, 2003; Diaconu et al., 2005; Kavran and Steitz, 2007). A fourth acidic P-protein (P3) has been identified in plants but to date no function has been attributed to it (BaileySerres et al., 1997). Together, L7/L12 (P1 and P2 in plants) form a flexible hinge that interacts with translation factor GTPase in mediating initiation, elongation, translocation and release of nascent polypeptides (BaileySerres et al., 1997; Diaconu et al., 2005; Kavran and Steitz, 2007). RPL10 (P0 in plants) binds the L7/L12 dimer to RPL11 (RPL12 in plants), which in turn binds to LSU rRNA (Ban et al., 2000; Gonzalo and Reboud, 2003; Kavran and Steitz, 2007). The P r-proteins are unique in two respects (1) two copies of each P1 and P2 are present in each ribosome and (2) following translation and phosphorylation in the cytoplasm, P r-proteins remain in the cytoplasm and are recruited to cytoplasmic ribosomes following LSU export into the cytoplasm

(Zinker and Warner, 1976; Sanchezmadrid et al., 1981; Elkon et al., 1986). Once phosphorylated, P r-proteins cycle between ribosomes and a cytosolic pool of P r-proteins never localizing to the nucleus/nucleolus (Zinker and Warner, 1976). My results support a possible similar situation for RPS15aB and E, the diffuse, weak cytosolic signal obtained from transient expression in tobacco epidermal cells could be a result of RPS15aB and E remaining in the cytoplasm for future addition to preformed SSUs.

While it has been suggested that RPS15aB and E are nuclear encoded mitochondrial r-proteins (Adams et al., 2002), several lines of evidence (along with the cellular localization reported here) suggest this not to be the case. The majority of plant mitochondrial proteins identified to date, contain a mitochondrial localization signal (MTS: presequence), comprised of a high degree of positively charged amino acids, that can form an amphiphilic  $\alpha$ -helix, in their N-terminus (Roise et al., 1986; Vonheijne, 1986; Hansel et al., 2002; Huang et al., 2009). The  $\alpha$ -helix binds to receptors on the mitochondrial membrane that then transport the protein into the mitochondria. During the transport process the presequence is cleaved and protein maturation occurs in the mitochondrion (Huang et al., 2009). RPS15aB and E do not have an N-terminal extension. In *Drosophila* (Frei et al., 2005), mice (Chen et al., 2007) and yeast (Saveanu et al., 2001) tagged mitochondrial r-proteins have successfully been used to document localization to mitochondria, therefore, the observation that RPS15aB and E were not found in mitochondria is likely due to the fact that they are not mitochondrial r-proteins rather than weak GFP expression in the fusion proteins.

The cellular localization studies reported here for RPS15aB and E show that they are not localized to the nucleus, nucleolus or mitochondria and the RNAi KD lines indicate that RPS15aB and/or E are required for normal plant growth and development. So, are type II isoforms located in the cytoplasm where they possibly form an active pool of RPS15aB and E, similar to the acidic P1 and P2 r-proteins, for delayed addition to preformed SSUs? While no RPS15aB or E was detected by western blot or LC-MS/MS analysis in fifteen-day-old *Arabidopsis* plants, expression profiles (Genevestigator) for RPS15aB and E transcript show highest transcript abundance during germinating seedlings. Coupled with constitutive RNAi type II family KDs that were lethal, these data

suggest RPS15aB and E may be specifically required during seed germination and therefore, not present at detectable levels in fifteen-day-old plants. Probing total protein extract from germinating seeds may help determine if this is in fact the case. It is important to note, however, that transcript levels can vary widely compared to protein levels. In yeast, transcript levels can vary 30 fold from their respective protein levels (Gygi et al., 1999; Zanetti et al., 2005) due to highly regulated and specific mRNA translation and protein degradation processes (Gutierrez et al., 1999; Hellmann and Estelle, 2002; Kawaguchi and Bailey-Serres, 2002).

Nuclear/nucleolar localization of r-proteins has been documented in yeast (Moreland et al., 1985; Rutgers et al., 1990; Schaap et al., 1991), human (Ko et al., 2006; Chou et al., 2010) and *Xenopus* (Claussen et al., 1999) and to some degree in plants (Raghavendra, 2011). Usually NLSs are located in the N terminal region of the cargo protein, it was therefore somewhat surprising to see the outcome of the C-terminal deletion analysis of RPS15a A/F and D as the last seven (or less) C-terminal amino acids are required for nuclear localization. One of the following three scenarios may be responsible for the lost nuclear localization; (1) the truncated protein is recognized as damaged and rapidly degraded by the 26S proteasome, (2) the lost lysine residue disrupts an as yet to be identified linear NLS and (3) the lost lysine residue acts in concert with upstream basic residues to form a 3D NLS, that upon folding, is lost. In both scenarios 2 and 3 disruption of a NLS would render the r-protein unable to bind to an importin, an association required for the shuttling of proteins through the NPC via the importin  $\beta$  pathway (Chook and Blobel, 2001; Pemberton and Paschal, 2005). To further investigate these seven C-terminal amino acids in nuclear localization a series of deletions should be generated in which each of the seven amino acids is removed or mutated and subcellular localization documented. To investigate if K<sup>124</sup> (amino acid one of the seven removed) is part of a NLS, site directed mutagenesis could be used to replace K with a non-basic amino acid, therefore, resulting in a change of charge without altering the size of the protein. These deletions/mutations would demonstrate the degree to which each of the seven C-terminal acids contribute to nuclear localization.

In an attempt to determine the requirement of individual *RPS15a* gene family members for normal plant growth and development, RNAi KDs were generated for each



member. Transcript abundance for all five active members of the family (*RPS15aA/D/F/B* and *E*) was measured in each KD line to identify if compensation by any one isoform occurred in response to a decreased expression of the KD isoform. In yeast, reduced levels of the acidic r-protein YP2 $\beta$  reduced expression of YP1 $\beta$  while reduced levels of YP1 $\beta$  increased the expression of YP2 $\beta$ , suggesting that expression of two of the r-proteins comprising the flexible lateral stalk regulate expression of each other (Bermejo et al., 1994; Remacha et al., 1995). However, the individual KD lines of Arabidopsis *RPS15a* were inconclusive. A consistent reduction in the expression of the targeted gene was not achieved and an inconsistent reduction or overexpression of non-target *RPS15a* genes was seen. These inconsistencies are probably the result of using a constitutive RNAi vector, such that, if strong KDs were lethal at the embryo stage then the obtained transgenics would be the result of T-DNA insertions into regions of the genome with low transcriptional activity or into genes involved in other unrelated pathways. To circumvent these outcomes, individual KDs should be generated using an inducible vector, such as pER8 (estrogen inducible vector) to control timing of expression of the RNAi cassette (Zuo et al., 2000; Guo et al., 2003; Degenhardt and Bonham-Smith, 2008). Root analysis of the RNAi lines did reveal a consistent mutant phenotype common to many r-protein mutants. All RNAi lines exhibited delayed primary root growth. Unlike many r-protein mutants, where a decrease in expression of the dominant paralog has resulted in the *pfl* phenotype and aberrant leaf venation (Weijers et al., 2001; Horiguchi et al., 2011; Horiguchi et al., 2012), the overall decrease in plant growth of the *RPS15a* RNAi lines, without subsequent leaf and venation abnormalities suggests that the decrease in the r-protein isoform levels were sufficient enough to effect plant growth, but insufficient to effect leaf development.

Due to the complexities of the *RPS15a* gene family, a more rigorous approach must be taken to investigate the requirements for type I and II isoforms. Not only individual, but multiple double and triple mutants must be generated to develop a comprehensive understanding of the requirements for each member of this family in normal plant growth and development. Of the *RPS15a* gene family members, type II members have received the least amount of interest and thus, exploration. Immunopurification of tagged RPS15aB and

E isoforms from Arabidopsis should provide important and definitive information as to the destination and function of these isoforms.

## **CHAPTER 2. CHARACTERIZING THE *RPS15A* GENE FAMILY – Relationship to the thesis in its entirety**

Chapter 2 relates to the thesis as it provides direct examples of how disruptions of r-proteins affect Arabidopsis growth and development. Knockdowns of either type I or II families was lethal while individual KDs inhibited root growth in all RNAi lines.

## CHAPTER 3. USING NEXT GENERATION SEQUENCING TO ANALYZE *pfl1* (*rps18a*) AND *pfl2* (*rps13a*) RIBOSOMAL PROTEIN MUTANTS

Mutations in a number of genes involved in ribosome biogenesis, miRNA biogenesis and auxin homeostasis have resulted in the *pointed first leaf* (*pfl*) phenotype suggesting that a common pathway is being disrupted in all three processes. Degenhardt and Bonham-Smith, (2008) suggested that this relationship is the result of an intricate pathway in which efficient ribosome biogenesis is required for miRNAs to effectively target the degradation of some auxin response factor (ARF) mRNAs otherwise required for normal leaf/plant development. Therefore, perturbations in any one of these three pathways would result in a similar phenotype. I had both the transcriptome and miRNOME of two r-protein mutants (*pfl1* (*rps18a*) and *pfl2* (*rps13a*)) sequenced using Illumina sequencing technology. Transcriptome analysis revealed 20 genes that were up or down regulated in both r-protein mutants compared to WT. Most of these 20 genes could be grouped into one of four categories, (1) plant defense (largest group), (2) transposable elements, (3) nitrogen metabolism, or (4) unknown functions. Of the 20, no genes were involved in miRNA biogenesis or auxin homeostasis with only one gene (*RIBOSOMAL PROTEIN 1: encodes RPL3A*) involved in ribosome biogenesis. To determine if the same genes were up and/or down regulated in auxin mutants *transport inhibitor response1* (*tir1*), *auxin signaling F-box protein1* (*afb1*), *auxin signaling F-box protein2* (*afb2*) and *auxin signaling F-box protein3* (*afb3*) were analyzed by qRT-PCR for 14 of the 20 genes identified in the Illumina sequencing. No gene-response similarities were found between the r-protein and auxin mutants or among the auxin mutants for the 14 genes investigated. The miRNOME of the two mutants was also sequenced to determine whether aberrant ribosome biogenesis altered the miRNA pools, therefore, disrupting auxin homeostasis. No miRNAs were found to be up or down regulated compared to WT. My data suggest that mutations in genes involved in ribosome biogenesis, miRNA biogenesis and auxin homeostasis result in similar phenotypes but through different pathways.

### 3.1 Introduction

Many r-protein mutants display a *pointed first leaf* (*pfl*) phenotype in their first to fourth leaves (in young plants), accompanied by a decrease in overall plant mass, abnormal

root morphology, delayed onset of flowering and a prolonged life cycle (Vanlijsebettens et al., 1994; Ito et al., 2000; Degenhardt and Bonham-Smith, 2008; Byrne, 2009). Looking at these mutant phenotypes independently from other unrelated but similar mutant phenotypes one could suggest that each is the result of decreased translational efficiency of the ribosome. Mutated r-proteins would decrease the number of functional ribosomes per cell, therefore, reducing the capacity of the cell to meet protein requirements, resulting in the pleiotropic phenotypes associated with these r-protein mutants. The similar phenotypes of miRNA biogenesis and auxin synthesis and transport mutants add complexity to this model. Degenhardt and Bonham-Smith (2008) postulated a pathway linking auxin homeostasis and ribosome biogenesis via micro RNAs (miRNAs) to explain the common mutant phenotypes. The pathway was suggested based on r-protein mutants (*rpl28a*, *rpl5a*, *rpl10a*, *rpl9*, *rpl5*, *rps5b*, *rps13a*, *rps18a*, *rpl24b* and *rpl23aa*) (Vanlijsebettens et al., 1994; Ito et al., 2000; Weijers et al., 2001; Nishimura et al., 2005; Degenhardt and Bonham-Smith, 2008; Imai et al., 2008; Pinon et al., 2008; Yao et al., 2008; Fujikura et al., 2009), auxin-responsiveness/polar auxin transport mutants (*ettin/auxin response factor 3* (*arf3*), *monopteros/arf5*, *pin-formed1*, *auxin-resistant1* (*axr1*), *hve1/cand1*, *lop1/tornado1* and *tornado2*) (Goto et al., 1991; Bennett et al., 1995; Carland and McHale, 1996; Cnops et al., 2000; Nemhauser et al., 2000; Adams et al., 2002; del Pozo et al., 2002; Deyholos et al., 2003; Cnops et al., 2006; Petrasek et al., 2006) and miRNA biogenesis mutants (*dcl1*, *hyl1*, *hen1* and *arg1*) (Bohmert et al., 1998; Jacobsen et al., 1999; Lu and Fedoroff, 2000; Chen et al., 2002), all resulting in abnormal leaf morphology (*pfl* phenotype) and aberrant leaf venation (open loop veins and decreased higher order venation).

Disrupting multiple aspects of auxin homeostasis (synthesis and transport) show similar abnormal venation patterns as r-protein and ribosome biogenesis mutants. The auxin regulated transcription factor mutants *arf3* and *arf5* both show aberrant leaf venation patterning similar to that found in ribosome biogenesis mutants (Przemeck et al., 1996; Sessions et al., 1997; Nemhauser et al., 2000; Degenhardt and Bonham-Smith, 2008). *PIN-FORMED* (*PIN*) genes encode proteins involved in cellular auxin efflux and mutants (e.g. *pin-formed1*) in these genes generate severely abnormal leaves characterized by narrow, twisted laminae and wide, branched midveins (Goto et al., 1991; Bennett et al., 1995; Petrasek et al., 2006). Mutations in several aspects of the SCF<sup>TIR</sup> ubiquitin ligase

complex also produce similar phenotypes. When bound to auxin, the SCF<sup>TIR</sup> complex promotes ubiquitination of Aux/IAA repressors and subsequent degradation of these repressors via the 26S proteasome. This releases the ARF family of transcription factors from Aux/IAA transcriptional repression allowing ARFs to bind to and activate auxin response elements (AREs), in the promoters of auxin-regulated genes (Guilfoyle et al., 1998; Parry et al., 2009). ARFs induce several principle groups of genes including *AUX/IAA*, *GH3* and *SMALL AUXIN-UP RNA (SAUR)*, as well as, various families of transcription factors; HD-Zip family, AP2-type, As2-like (LBD), MYB-like and zinc finger-like (Guilfoyle and Hagen, 2007; Chapman and Estelle, 2009).

Mutations in a number of genes involved in the SCF<sup>TIR</sup> pathway can result in leaves with abnormal venation. When mutated, *AXR1*, that encodes a protein that forms a heterodimer with E1 C-TERMINAL RELATED 1 (ECR1) (Hotton et al., 2011) required to activate SCF ubiquitin protein ligase, results in plants with smaller, slightly pointed rosette leaves, reduced leaf venation and a loss in apical dominance (del Pozo et al., 2002; Deyholos et al., 2003). *HEMIVENATA1 (HVE1/CAND1)* encodes the cullin associated and neddylation dissociated (CAND1) protein that, in mammals, regulates formation of the SCF complex by preventing the association between CUL1 and SKP1/SKP2 (CUL1 binds to SKP1 and SKP1 interacts with F box proteins) (Zheng et al., 2002; Alonso-Peral et al., 2006). Mutation of *CAND1* in plants results in leaves with reduced secondary and tertiary veins suggesting that CAND1 is involved in regulating the auxin response through the SCF ubiquitin ligase complex (Alonso-Peral et al., 2006). *LOP1/TORNADO1* and *TORNADO2* encode proteins with unknown functions in auxin perception/signaling but are essential for normal leaf development. Mutations in either gene lead to extremely narrow leaves with severely reduced leaf venation (Carland and McHale, 1996; Cnops et al., 2006). The high degree of phenotypic similarity between r-protein mutants and these mutants in multiple aspects of auxin synthesis and distribution is highly suggestive that some common element connects ribosome biogenesis and auxin synthesis and transport in the cell. Endogenous short regulatory non-coding RNAs (miRNAs) have been implicated as possible links connecting these two processes (Eulalio et al., 2007).

miRNAs are small (21-23 nucleotide) RNAs generated from long single stranded endogenous RNAs, that upon transcription, fold back on themselves (due to internal

complementarity) to form hairpin structures. miRNAs, processed from these hairpins by DICER-LIKE enzymes (DCL1) and double stranded RNA binding proteins HYPONASTIC LEAVES1 (HYL1), are methylated by HUA ENHANCER1 (HEN1), before being loaded onto one of the ten ARGONAUTE (AGO) proteins in Arabidopsis. AGOs facilitate the binding and degradation of target transcripts (or translational repression) identified by the guide strand miRNA that shares near perfect complementarity with the target transcript (Mallory and Vaucheret, 2006). In *Drosophila*, targeting of some miRNA transcripts was shown to be translation dependent; reducing ribosome function resulted in the stabilization of miRNA-targeted proteins (Eulalio et al., 2007). Eulalio and coworkers (2007) demonstrated the dependence of miRNA targeting of transcripts on translation by fusing the 3' UTR of known miRNA targets to the firefly luciferase ORF and quantifying luciferase transcript levels, both in the presence and absence of miRNAs. In the presence of translation inhibitors some transcript levels, that previously had decreased significantly in the presence of miRNAs, stabilized suggesting that active translation was required for miRNA-mediated degradation (Eulalio et al., 2007). In plants, many miRNA-targeted transcripts are involved in the auxin response pathway, e.g., TIR1, a component of the ubiquitination pathway regulated by miR393 (Navarro et al., 2006), NAC1/NAM, involved in auxin signaling, ARF2, ARF3 and ARF4, miR390-dependent TAS3siRNA regulated, ARF6 and ARF8, miR167 regulated, ARF10, ARF16 and ARF17, auxin response factors regulated by miR160 (Bonnet et al., 2004; Vazquez et al., 2004; Xie et al., 2007; Meng et al., 2010; Jay et al., 2011; Sunkar et al., 2012). It has been proposed that stabilization of these transcripts could result in destabilization of the auxin feedback cycle leading to the aberrant phenotypes associated with r-protein, auxin response and miRNA mutants (Degenhardt and Bonham-Smith, 2008).

Abiotic and biotic stresses such as drought, salt, ABA, UVB, cold, heat and bacterial infection have all been shown to alter miR160, miR167 and miR393 expression, each of which target many ARFs (Fahlgren et al., 2007; Zhang et al., 2011; Sunkar et al., 2012). These observations support the suggestion that a cell could perceive a decrease in ribosome biogenesis (due to r-protein mutants) as a cellular stress and modulate miRNA expression accordingly, thus, linking ribosome biogenesis, miRNA biogenesis and auxin synthesis and transport pathways to the resulting mutant phenotypes.

Here (using Illumina next generation sequencing technology) I present data detailing changes in both the transcriptome and miRNOME of two *Arabidopsis* r-protein mutants (*pfl1* (*rps18a*) and *pfl2* (*rps13a*)). Results obtained suggest that the pathway postulated by Degenhardt and Bonham-Smith (2008) in which miRNAs regulate ARFs in a translation dependent manner is more complex than first suggested and that the common phenotypes seen in ribosome biogenesis, miRNA biogenesis and auxin synthesis and transport mutants may in fact arise through unique pathways.

### **3.2 Materials and Methods**

#### **3.2.1. Plant material and growth conditions**

Homozygous *Arabidopsis thaliana* mutants were obtained from the Arabidopsis Biological Resource Center (ABRC) (*pfl1*, *tir1*, *afb1*, *afb2* and *afb3*) and Rikagaku Kenkyūjo (RIKEN) (*pfl2*). All plants were grown under a 23°C/17°C temperature regime and 16/8 (light/dark) hour photoperiod of ~120  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Seeds were stratified in the dark for two days at 4°C before transfer to a Conviron growth chamber for 13 days. Thirteen-day-old plant tissue (whole plants) from WT, *pfl1*, *pfl2*, *tir1*, *afb1*, *afb2* and *afb3* was collected and flash frozen in liquid Nitrogen ( $\text{N}_{2(l)}$ ) before storage at -80°C.

#### **3.2.2. Sample preparation for Illumina sequencing**

Sample preparation for Illumina transcriptome and miRNOME sequencing was identical. Plant tissue stored at -80°C was pulverized in  $\text{N}_{2(l)}$  and total RNA was extracted from 13 day old WT, *pfl1* and *pfl2* plants using the Qiagen RNeasy Plant Minikit (Qiagen). RNA samples were assessed for quality (RNA Integrity Number (RIN) of at least 8.0 for all three samples to ensure maximum conversion of RNA to cDNA) and quantity using a Nanodrop 2000 (Thermo Scientific), packed on ice and immediately sent to the PBI-NRC (Saskatoon, SK) for Illumina sequencing. At PBI-NRC the RNA was converted to a cDNA library, fragmented and adenylated. Sequencing adaptors were ligated to the adenylated fragments, which were then ligated to the sequencing flow cell. Once bound to the flow cell, amplification and sequencing was carried out. Transcriptome sequencing (Illumina GAIIx) resulted in 36 base pair reads while the miRNOME sequencing (Illumina HiSeq) produced 100 base pair reads.



### 3.2.3. Analysis of raw FASTQ data

NRC-PBI (Saskatoon, SK) provided raw FASTQ files from the transcriptome and miRNOME sequencing runs and data analysis was carried out using NextGENe Version 2.15 (transcriptome) or Version 2.20 (miRNOME).

#### 3.2.3.1. Transcriptome analysis

Raw FASTQ files (WT - 41,534,445, *pfl1* - 21,960,475 and *pfl2* - 22,676,323 reads) were converted to FASTA files (median score threshold  $\geq 20$ , maximum number of uncalled bases  $\leq 3$ , called base number for each read  $\geq 25$ , trim or reject read when  $\geq 3$  bases with score  $\leq 16$ ) and sequencing adaptors (5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3') trimmed. Converted, trimmed sequences for WT, *pfl1* (*rps18a*) and *pfl2* (*rps13a*) were independently aligned to the Arabidopsis transcriptome using one round of condensation and one round of alignment (matching requirement  $\geq 12$  bases,  $\geq 80\%$  identity, mutation filter  $\leq 5$ , SNP allele  $\leq 1$  count, coverage  $\leq 20$ , forward/reverse balance  $\leq 0.05$  and read library size range of 50 – 300) using TAIR 10 (obtained from TAIR, 2011). Expression reports of WT, *pfl1* and *pfl2* were used as input values for DESeq statistical analysis (Section 3.2.4).

#### 3.2.3.2. miRNOME analysis

Raw FASTQ files (WT – 47,516,630, *pfl1* – 47,487,304 and *pfl2* – 52,590,275 reads) were converted to FASTA files (as described in Section 3.2.3.1). The poly A tail (sequence 'AAAAAA') associated with each read was trimmed followed by trimming of the sequencing adaptor (5-'ATCTCGTATGCCGTCTTCTGCTTG'-3'). The resulting reads for WT, *pfl1* and *pfl2* were independently aligned to the known Arabidopsis miRNOME (miRNOME obtained from MirBase: at time of analysis there were 287 miRNAs) (Griffiths-Jones et al.; Griffiths - Jones, 2004; Griffiths-Jones et al., 2008; Kozomara and Griffiths-Jones, 2011) with matching requirement  $\geq 12$  bases,  $\geq 50\%$  identity, mutation percentage  $\leq 20\%$ . Expression reports for WT, *pfl1* and *pfl2* were used as input values for DESeq statistical analysis.

#### 3.2.4. DESeq statistical analyses

DESeq is an R package (available through Bioconductor) to analyze count data from high-throughput sequencing assays to test for differential expression. I chose this statistical method as it allowed me to determine expression differences in the absence of replicates. DESeq assumes that the mean is still a good predictor for dispersion because we only expect minor changes in expression levels for the majority of the genes across our samples (WT, *pfl1* and *pfl2*). Therefore, we can take the dispersion estimated from comparing the counts across samples (WT, *pfl1* and *pfl2*) as *ersatz* for a proper estimate for the variance across replicates. As the differentially expressed genes will cause the dispersion estimate to be too high, the test will err on the side of being too conservative, therefore, genes identified as being up or down regulated will be highly significant but many genes without a large up or down swing in regulation will be missed (Anders and Huber, 2010).

#### 3.2.5. Quantitative RT-PCR

RNA extraction, first strand cDNA synthesis and qRT-PCR on 13-day-old WT, *pfl1*, *pfl2*, *tir1*, *afb1*, *afb2* and *afb3* were carried out as described in Section 2.2.4. To ensure *ACT7* abundance was not fluctuating in the mutants and was therefore an acceptable housekeeping gene, *ACT7* reads from the Illumina data were recorded and found to be consistent between WT, *pfl1* and *pfl2*.

#### 3.2.6. Statistics

Refer to section 2.2.10.

### 3.3 Results

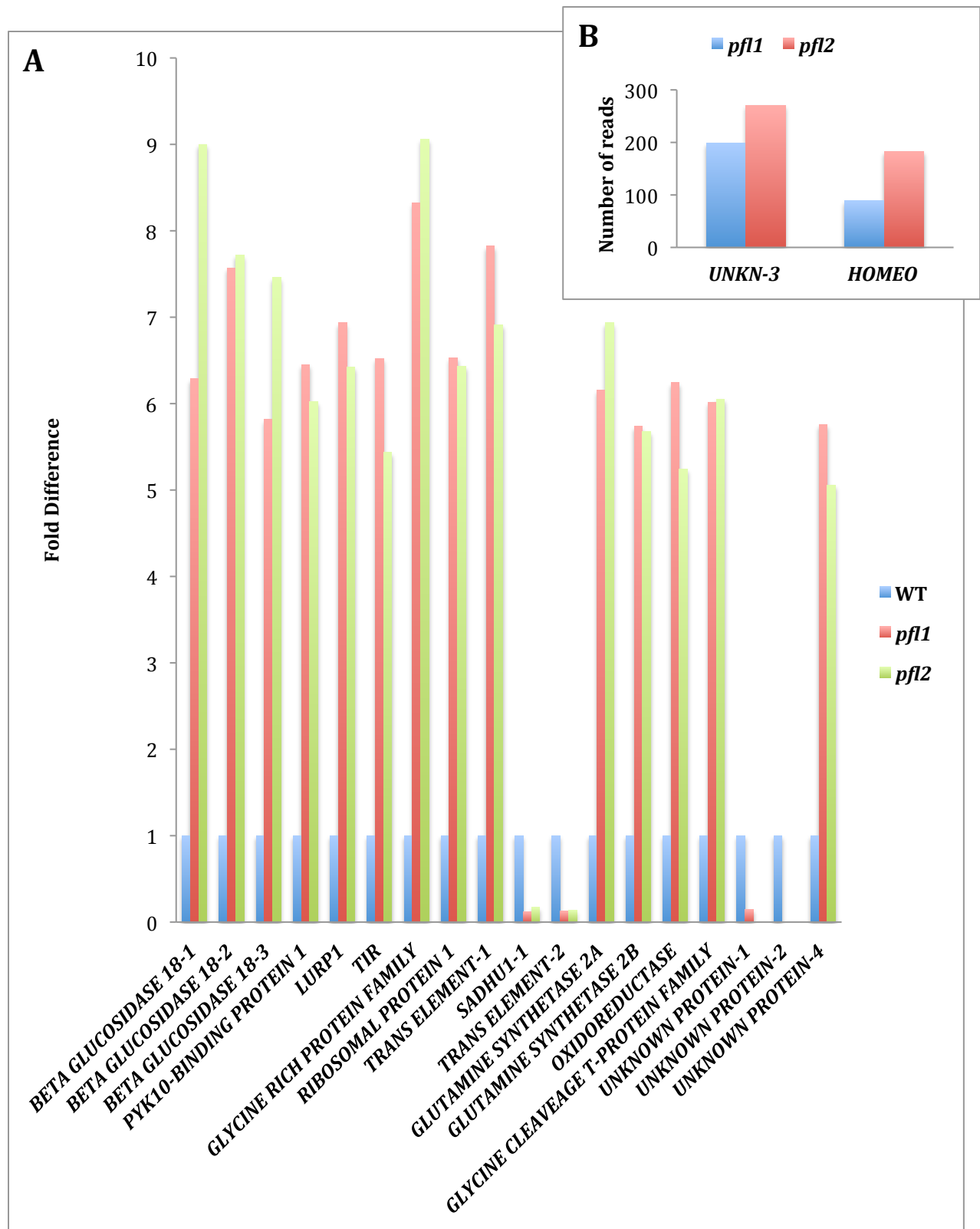
#### 3.3.1. Common changes in transcriptome in *pfl1* and *pfl2* compared to WT

To fully appreciate the degree to which r-protein mutants affect whole plant transcriptional status, next generation sequencing was carried out on two r-protein mutants, *pfl1* (*rps18a*) and *pfl2* (*rps13a*) and compared to WT. Transcripts of 20 genes were found to be commonly increased or decreased between *pfl1* and *pfl2* compared to WT (Table 3.1). Of the 20 genes, transcripts for 16 were increased (two unknown genes,

AT5G15360 and AT1G20280, not transcribed in WT were transcribed in both *pfl1* and *pfl2*) while transcripts for four were decreased (one unknown gene, AT5G49440, transcribed in WT was not transcribed in either *pfl1* or *pfl2* and a second unknown, AT4G34881, is decreased 6.9 fold in *pfl1* while no longer transcribed in *pfl2*) (Figure 3.1). My initial hypothesis stated that a number of auxin, miRNA and ribosome biogenesis genes would be among those deregulated in the r-protein mutants as all three types of mutants share similar phenotypes (Degenhardt and Bonham-Smith, 2008). Of these three gene groups only a single gene, *RIBOSOMAL PROTEIN 1*: encoding RPL3A, was identified as having a known role in ribosome biogenesis. Of the other increased or decreased transcripts, none have identified roles in auxin biosynthesis or miRNA biogenesis. Two (AT4G34881 and AT5G49440) of the five unknowns have orthologs in numerous prokaryotes and eukaryotes while the other three (AT5G15360, AT1G20280 and AT2G25510) are plant specific. All of the genes for which transcription was either switched on or off in the r-protein mutants have unknown functions. The importance of these five genes in plant growth and development is suggested by the lack of homozygous T-DNA lines at the ABRC, suggesting that, although their function is currently unknown, the encoded proteins may be required for normal plant growth and development.

**Table 3.1: List of the 20 transcripts and their cellular function from Illumina data showing increased or decreased expression levels in *pfl1* and *pfl2* compared to WT**

Gene Locus / Gene Name	Cellular Function	<i>pfl1</i>	<i>pfl2</i>
AT1G52400.3   <i>BETA GLUCOSIDASE 18-1</i>	Plant Defense	Up	Up
AT1G52400.1   <i>BETA GLUCOSIDASE 18-2</i>	Plant Defense	Up	Up
AT1G52400.2   <i>BETA GLUCOSIDASE 18-3</i>	Plant Defense	Up	Up
AT3G16420.1   <i>PBP1 - PYK10-BINDING PROTEIN1</i>	Plant Defense	Up	Up
AT2G14560.2   <i>LURP1</i>	Plant Defense	Up	Up
AT1G72930.1   <i>TOLL/INTERLEUKIN-1 RECEPTOR-LIKE</i>	Plant Defense	Up	Up
AT2G05530.1   <i>GLYCINE-RICH PROTEIN FAMILY</i>	rRNA Processing/Structural	Up	Up
AT1G43170.1   <i>RIBOSOMAL PROTEIN 1</i>	Ribosome Biogenesis	Up	Up
AT2G11240.1   <i>TRANSPOSABLE ELEMENT GENE-1</i>	Transposable Element	Down	Down
AT2G10410.1   <i>SADHU1-1</i> (Transposable element)	Transposable Element	Down	Down
AT4G08030.1   <i>TRANSPOSABLE ELEMENT GENE-2</i>	Transposable Element	Up	Up
AT5G35630.3   <i>GLUTAMINE SYNTHETASE 2A</i>	Nitrogen Metabolism	Up	Up
AT5G35630.2   <i>GLUTAMINE SYNTHETASE 2B</i>	Nitrogen Metabolism	Up	Up
AT1G20020.1   <i>FERRODOXIN-NADP(+)-OXIDOREDUCTASE 2</i>	Photosynthetic Enzyme	Up	Up
AT1G11860.3   <i>GLYCINE CLEAVAGE T-PROTEIN FAMILY</i>	Glycine Degradation	Up	Up
AT1G20280.1   <i>HOMEODOMAIN-LEUCINE ZIPPER PROTEIN-RELATED</i>	Unknown	Up	Up
AT4G34881.1   <i>UNKNOWN PROTEIN-1</i>	Unknown	Down	Down
AT5G49440.1   <i>UNKNOWN PROTEIN-2</i>	Unknown	Down	Down
AT5G15360.1   <i>UNKNOWN PROTEIN-3</i>	Unknown	Up	Up
AT2G25510.2   <i>UNKNOWN PROTEIN-4</i>	Unknown	Up	Up



**Figure 3.1. Transcript levels up or down regulated in *pfl1* and *pfl2*.** 20 Illumina identified transcripts up and/or down regulated in *pfl1* and *pfl2*. (A) Sixteen transcripts are up regulated in *pfl1* and *pfl2* compared to WT while four transcripts are down regulated. (B) The two transcripts, AT5G15360 and AT1G20280, inactive in WT are turned on in *pfl1* and *pfl2*, both genes encode proteins with unknown cellular functions.

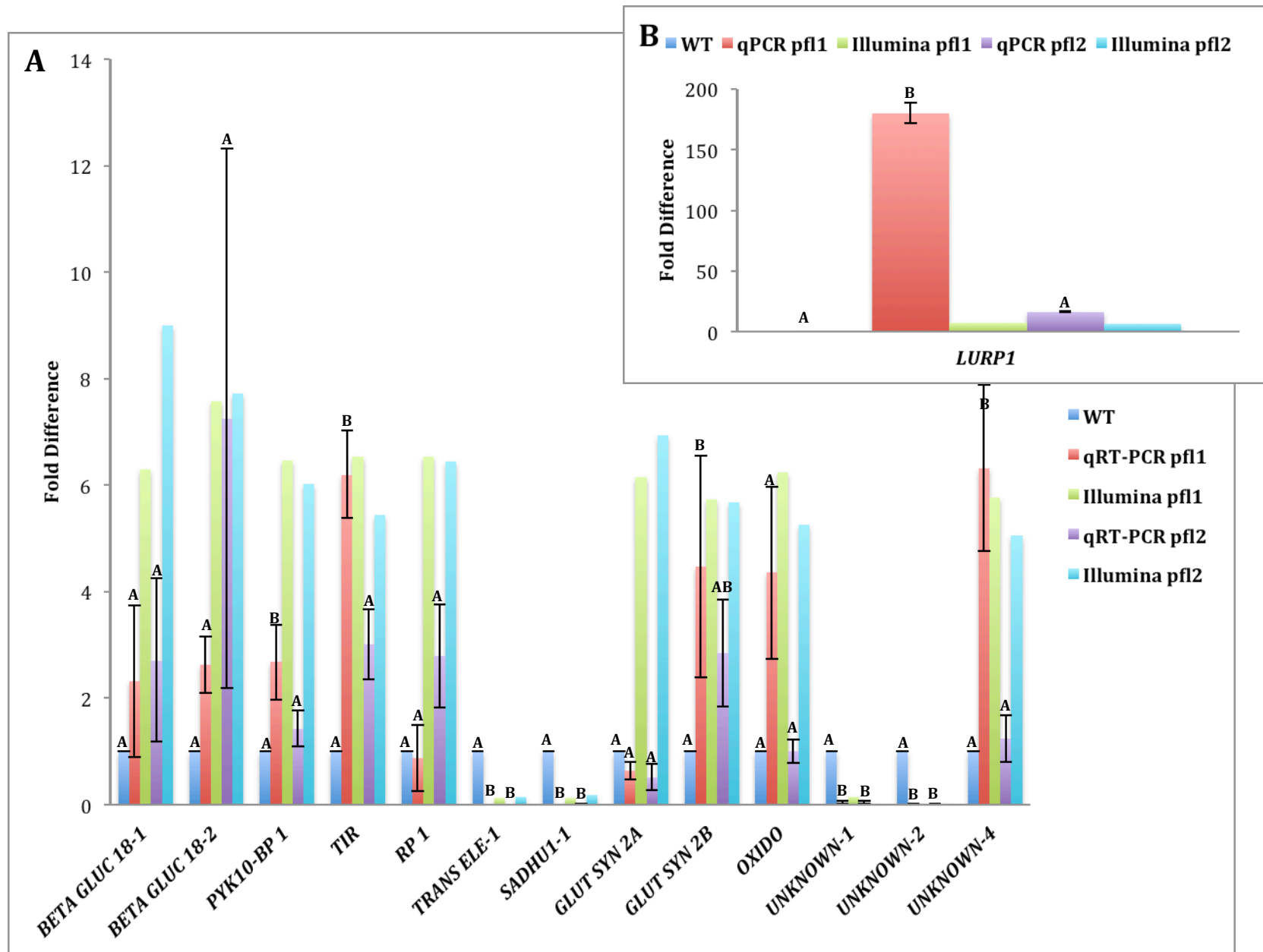
### **3.3.2. qRT-PCR and Illumina data show similar trends of up and/or down regulation of Illumina identified transcripts**

To determine the validity of the Illumina results, primers were designed for each of the 20 identified genes. However, discrete amplicons could only be obtained for 14 of the 20 genes. Primers could not be designed to obtain concise, single amplicons for *BETA GLUCOSIDASE 18-3*, *UNKNOWN PROTEIN-3*, *TRANSPOSABLE ELEMENT-2*, *HOMEBOX LEUCINE ZIPPER PROTEIN RELATED*, *GLYCINE RICH PROTEIN FAMILY* and *GLYCINE CLEAVAGE T-PROTEIN FAMILY*, due to these genes being members of large gene families sharing high sequence identity. qRT-PCR was carried out on the remaining 14 genes in 13 day old *pfl1* and *pfl2* plants. The trends for transcript levels were similar for 11 of the 14 genes, even though the absolute transcript levels varied between the Illumina and qRT-PCR data (Figure 3.2A and B). For the remaining three genes *pfl1* varied from trend for *RIBOSOMAL PROTEIN 1*, *pfl2* varied from trend for *OXIDOREDUCTASE* and both varied from trend for *GLUTAMINE SYNTHETASE 2A*. A discrepancy in the trend may be due to the use of a single sample for the generation of the Illumina data; whereas, the qRT-PCRs were carried out in triplicate.

### **3.3.3. Transcript levels for Illumina-identified genes in *tir1*, *afb1*, *afb2* and *afb3* auxin mutants show little similarity to those in *pfl1* and *pfl2***

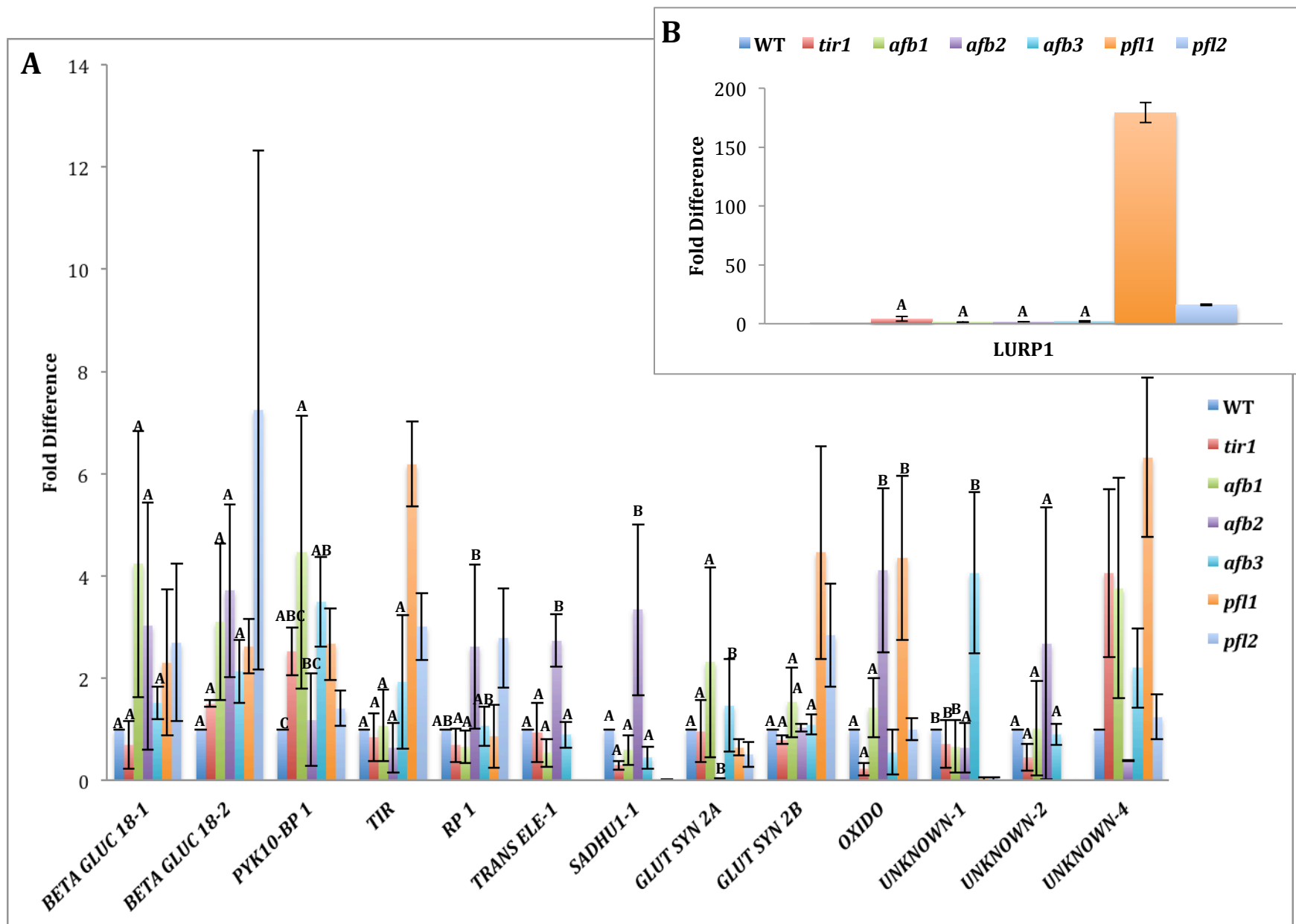
My initial hypothesis states there is a link between auxin biosynthesis, miRNA biogenesis and ribosome biogenesis based on similar mutant phenotypes; therefore, four auxin mutants were examined to determine if genes up or down regulated in *pfl1* and *pfl2* were also deregulated in the auxin mutants *tir1*, *afb1*, *afb2* and *afb3*. TIR1 and AFB are F-box proteins in ubiquitin ligase complexes that when bound to auxin, promote the degradation of transcriptional repressors, therefore, promoting the transcription of auxin response factors (ARFs) (Parry et al., 2009). qRT-PCR of the 14 Illumina identified deregulated transcripts in *pfl1* and *pfl2* was performed on tissue from 13-day-old *tir1*, *afb1*, *afb2* and *afb3* plants.

*BETA GLUCOSIDASE 18-1* transcript levels decreased 1.4 fold in *tir1* but increased in the *afb* and *pfl* mutants with *afb1* showing the greatest up regulation (4.2 fold). *BETA GLUCOSIDASE 18-2* and *PYK10-BINDING PROTEIN1* showed a similar increased transcript





**Figure 3.2. Illumina and qRT-PCR results for the fourteen Illumina identified transcripts.** (A) qRT-PCR and Illumina results for 13 of the 14 transcripts in *pfl1* and *pfl2*. (B) qRT-PCR and Illumina results for *LURP1*. A Tukey test was carried out between each of the plant lines. Means of 3 biological replicates per line ( $\pm$  standard error) with same letters do not differ significantly ( $P \leq 0.1$ ). Three technical replicates were performed for each RNA sample. Analysis is only between mutants for each Illumina gene.



**Figure 3.3. qRT-PCR of auxin and r-protein mutants of the fourteen Illumina identified transcripts.** (A) qRT-PCR for 13 of the 14 transcripts in four auxin mutants. (B) qRT-PCR of *LURP1* in both r-protein and auxin mutants. A Tukey test was carried out between each of the plant lines. Means of 3 biological replicates per line ( $\pm$  standard error) with same letters do not differ significantly ( $P \leq 0.1$ ). Three technical replicates were performed for each RNA sample. Statistical analysis was carried out on auxin mutants only, *pfl* mutant statistics analysis is presented in figure 3.2. Analysis is only between mutants for each Illumina gene.

level in all mutants, with *pfl2* at 7.2 fold showing the greatest increase, as was also the case with *BETA GLUCOSIDASE 18-1*. *afb1* showed the greatest increase in *PYK10-BINDING PROTEIN1* (4.5 fold). *TOLL/INTERLEUKIN-1 RECEPTOR-LIKE* decreased in *tir1* and *afb2* plants and was upregulated in *afb1*, *afb3*, *pfl1* and *pfl2*, showing the greatest increase in *pfl1* (6.2 fold). *RIBOSOMAL PROTEIN1* decreased in *tir1*, *afb1* and *pfl1*, was unchanged in *afb3* and was upregulated in *afb2* and *pfl2*. *TRANSPOSABLE ELEMENT1* and *SADHU1-1* (transposable element) exhibited similar expression levels in auxin and r-protein mutants. *tir1*, *afb1* and *afb3* all showed decreased transcript levels, *afb2* had increased levels and in *pfl1* and *pfl2* both genes were turned off. *GLUTAMINE SYNTHETASE 2A* and *GLUTAMINE SYNTHETASE 2B* transcript levels varied amongst the mutants examined and were not coordinated within a single mutant. Reduced transcript abundance of *GLUTAMINE SYNTHETASE 2A* was recorded in *afb2*, *pfl1* and *pfl2*, increased in *afb1* and *afb3* and there was no change in *tir1*. *tir1* was the only mutant line to show a decrease in *GLUTAMINE SYNTHETASE 2B* while in *afb1*, *pfl1* and *pfl2* lines, transcript abundance increased. WT levels of *GLUTAMINE SYNTHETASE 2B* were recorded in *afb2* and *afb3*. In *afb1*, *afb2* and *pfl1*, *FERRODOXIN-NADP(+)-OXIDOREDUCTASE 2* was upregulated, 1.4, 4.1 and 4.3 fold, respectively. *tir1* and *afb3* plants showed decreased levels while in *pfl2* there was no change. In all but *afb3* (increased 4 fold), *UNKNOWN PROTEIN1* was decreased, most substantially in *pfl1* (27 fold) and *pfl2* (26 fold). There was a strong reduction of *UNKNOWN PROTEIN2* in *pfl1* (125 fold) and *pfl2* (270 fold) while in *afb1* and *afb3* lines, transcript levels were close to WT. *afb2* showed a 2.7 fold increase and *tir1* a 2.2 fold decrease. In all but *afb2* (2.6 fold decrease), *UNKNOWN PROTEIN4* transcript levels increased. In all mutant lines investigated, *LURP1* was upregulated with the largest increase in *pfl1* (179 fold).

No consistent pattern of transcript levels was observed between the auxin and *pfl* mutants for the 14 genes analyzed. However, other than *tir1*, all mutants did show increased transcript levels for the three defense genes *BETA GLUCOSIDASE 18-1*, *BETA GLUCOSIDASE 18-2* and *PYK10 BINDING PROTEIN1*. Two of the plant defense genes, *BETA GLUCOSIDASE 18-2* and *PYK10 BINDING PROTEIN1*, were the only 2 genes to show similar transcript levels among the auxin mutants. Between the *afb* mutants some similar patterns were observed. Among all three *afb* mutants, not including the plant defense genes, only

one gene, *GLUTAMINE SYNTHETASE 2B*, was commonly upregulated. In *afb1* and *afb2*, *GLUTAMINE SYNTHETASE 2B*, *OXIDOREDUCTASE* and *UNKNOWN2* was upregulated and *UNKNOWN1* was down regulated. Between *afb1* and *afb3*, six genes showed similar transcript levels. *TOLL/INTERLEUKIN-1*, *GLUTAMINE SYNTHETASE2A*, *GLUTAMINE SYNTHETASE2B* and *UNKNOWN4* were upregulated while *TRANSPOSABLE ELEMENT-1* and *SADHUA* (both transposable elements) were down regulated. *RIBOSOMAL PROTEIN 1* and *GLUTAMINE SYNTHETASE 2B* were the only two genes to exhibit a similar expression pattern in *afb2* and *afb3*. In all but *RIBOSOMAL PROTEIN1*, the *pfl* mutants showed a consistent pattern of transcript levels.

No strong correlation between the 14 deregulated transcripts was observed between the r-protein and auxin mutants (Figure 3.3). As well, little similarity between the gene expression patterns of the same 14 transcripts in the four auxin mutants was observed (Figure 3.3). However, with only one sample for each of *pfl1* and *pfl2*, the Illumina sequencing and subsequent data analysis may have been unsuccessful in identifying those genes involved in any common pathway with subtleties in the transcriptome overlooked during the statistical analysis of single events.

#### **3.3.4. miRNOME analysis of *pfl1* and *pfl2* showed no change in miRNA pools**

To determine if active translation, synonymous with normal ribosome biogenesis, affects the prevalence of miRNAs, the miRNOMEs (micro RNA genome) of 13-day-old WT, *pfl1* and *pfl2* plants were sequenced through 100 base pair Illumina reads (NRC-PBI Saskatoon, SK). Following statistical analysis using DESeq (Bioconductor), no miRNAs were found to be up or down regulated in *pfl1* or *pfl2* suggesting that perturbations in r-protein pools (*RPS18A* and *RPS13A*) did not effect the currently known miRNA population.

### **3.4 Discussion**

An elegant model was proposed by Degenhardt and Bonham-Smith (2008) in which it was suggested that active translation was required for efficient miRNA mediated transcript degradation of a variety of auxin related genes (e.g. *TIR1*, *NAC1/NAM*, *ARF6*, *ARF8*, *ARF10*, *ARF16* and *ARF17*) (Bonnet et al., 2004; Vazquez et al., 2004; Mallory and Vaucheret, 2006; Xie et al., 2007). The pathway was proposed in an attempt to explain the

similar morphological phenotypes associated with gene mutations affecting ribosome biogenesis, miRNA biogenesis and auxin synthesis and transport. Here I show that the suggested pathway is incomplete in its approach to the relatedness of the three processes and that the similar mutant phenotypes documented within each of these processes may be coincidental.

The transcriptomes of 13 day old WT, *pfl* and *pfl2* mutants were sequenced in an attempt to identify genes similarly up or down regulated and responsible for generating the *pfl* phenotypes. Twenty such genes, involved in plant defense, nitrogen metabolism, transposable elements and a large group of genes of unknown functions, were identified in both *pfl1* and *pfl2*. Two  $\beta$ -GLUCOSIDASEs were identified in this study,  *$\beta$ -GLUCOSIDASE 18* and PYK10-binding protein 1 (PBP1). Both members are of the glycoside hydrolase family 1 that in Arabidopsis is comprised of 47 members, all of which function mainly in plant defense (Rask et al., 2000; Lipka et al., 2005; Nagano et al., 2005; Nagano et al., 2008). The majority of the  $\beta$ -GLUCOSIDASE family localize to endoplasmic reticulum (ER) bodies, ER derived compartments surrounded by ribosomes and involved in plant defense against herbivory (Matsushima et al., 2003; Matsushima et al., 2004; Kimi et al., 2009), that are most prevalent in seedlings and mature roots or established in rosette leaves following mechanical wounding or an application of methyl jasmonate (Matsushima et al., 2002). PBP1 does not localize to ER bodies but instead is found in the cytoplasm where it functions as a molecular chaperon to ensure proper polymerization of PYK10 in response to mechanical or pathogen tissue damage (Nagano et al., 2005).

Similarly, LURP1 and TOLL/INTERLEUKIN-1 are also involved in the plant response to pathogen infection. *LURP1 (LATE UPREGULATED IN RESPONSE TO HYALOPERONOSPORA PARASITICA)* is upregulated when plants are exposed to the oomycete pathogen *Hyaloperonospora parasitica*, with this response being mediated by Resistance (R) proteins RPP4 and RPP7 (Eulgem et al., 2004; Eulgem and Somssich, 2007; Knoth et al., 2007). TOLL/INTERLEUKIN-1 is a nucleotide-binding domain and leucine-rich repeat (NB-LRR) type receptor involved in innate immunity in plants (Chisholm et al., 2006; Wirthmueller et al., 2007). Activation of these proteins results in transcriptional activation of downstream genes responsible for basal cellular defense and programmed

cell death surrounding the point of receptor activation (Tao et al., 2003; Chisholm et al., 2006; Takken et al., 2006; Wirthmueller et al., 2007). In *pfl1* (*rps18a*) and *pfl2* (*rps13a*) it is possible that a decreased ribosome biogenesis may be perceived as a cellular stress, much like a biotic or abiotic stress, resulting in a modulated plant activity. I suggest that this “artificial” stress (plants have not selected for a natural response to genetic manipulation) is recognized by the plant as a pathogen infection and as such the plant mounts a pathogen response.

Of the three transposable elements identified through Illumina sequencing two, *TRANSPOSABLE ELEMENT-1* and *SADHU1-1*, were down regulated and *TRANSPOSABLE ELEMENT-3* was up regulated in both *pfl1* and *pfl2*. In the r-protein mutants, *SADHU1-1* expression was reduced 5.8 to 8.4 fold. A similar decrease in expression has also been reported in Arabidopsis in response to haustoria formation by the obligate fungal biotroph *Golovinomyces cichoracearum*, although no transposable element mediated mechanism has been suggested (Fabro et al., 2008). In Arabidopsis, the expression of a variety of genes involved in pathogen defense, wounding, trichome development and senescence are regulated by the WRKY zinc-finger-type transcription factor family (Miao et al., 2004). A senescence-specific member of the family, WRKY53, has been shown to regulate expression of the transposable element AT4G08030.1 (Hinderhofer and Zentgraf, 2001), therefore, it is possible that other WRKY family members involved in plant defense may do so through the regulation of an array of transposable elements. Although deregulation of transposable elements in response to pathogen infection coincides with the other genes identified by Illumina sequencing (*β-GLUCOSIDASE 18*, *PBP1*, *LURP1* and *TOLL/INTERLEUKIN-1*) no direct link to ribosome biogenesis, miRNA biogenesis and auxin homeostasis is obvious.

It is difficult to ascribe any role for proteins with unknown functions in a model connecting the three processes of ribosome biogenesis, miRNA and auxin function. Two of the unknown genes (*UNKNOWN1* and *UNKNOWN2*) are present in all domains of life while the other two (*UNKNOWN3* and *UNKNOWN4*) are plant specific. *UNKNOWN1* and 2 are either turned off or have decreased expression in *pfl1* and *pfl2* while the plant specific genes (*UNKNOWN3* and 4) are turned on or have increased expression in the r-protein

mutants. Currently, only heterozygous T-DNA-tagged lines for each of the five unknown genes are available from ABRC. With homozygous lines, qRT-PCR of the 14 identified genes could be performed to see if a common subset of genes is up or down regulated in both the unknown T-DNA-tagged lines and the r-protein mutants. The optimal scenario would be for the homozygous T-DNA-tagged unknown lines to be sequenced by Illumina technology and the cellular transcriptional status to be compared to that of the r-protein mutants.

In contrast to animals in which ~ 60% of protein-coding genes are regulated by miRNAs (Friedman et al., 2009), plant miRNAs target a relatively small portion of transcriptionally active genes (Addo-Quaye et al., 2008; German et al., 2008; Li et al., 2010; Rubio-Somoza and Weigel, 2011). However, transcription factors and F-box proteins comprise a large number of these targets, thus, while having relatively few primary targets, plant miRNAs can regulate a large array of downstream plant genes due to the cascade effect (Rhoades et al., 2002; Jones-Rhoades and Bartel, 2004; Jones-Rhoades et al., 2006; Chen et al., 2010).

As previously mentioned, expression levels of miRNAs can be regulated by a multitude of abiotic and biotic stresses, resulting in increased or decreased regulation of their target sequences. To determine if ribosomal stress (decreased ribosome biogenesis) was perceived as an abiotic stress, the miRNOME of *pfl1* and *pfl2* was sequenced. No differences in miRNA expression levels were found between *pfl1*, *pfl2* and WT but this may be an artifact of using a single replicate, as was the case with the transcriptome data. The lack of replicates in the miRNOME sequence analysis may have resulted in subtle changes in the miRNOME between the mutants and WT being overlooked and possible connections between miRNAs and ribosome biogenesis missed. Repeating this experiment with the appropriate number of replicates would remove such ambiguity.

Little correlation of expression patterns among the four auxin mutants and the two *pfl* mutants, suggests that the 14 genes up and down regulated in the two r-protein mutants are not responsible for the similar phenotypes found in auxin and *pfl* mutants. When looking at the Illumina identified genes in the four categories identified earlier, we see some trends. From the  $\beta$ -glucosidase family, most mutant lines (auxin and r-protein) show an increased expression of *BETA GLUCOSIDASE 18-1*, *BETA GLUCOSIDASE 18-3* and *PYK10*-



*BINDING PROTEIN*, the only exception is seen in the *tir1* mutant in which *BETA GLUCOSIDASE 18-1* is down regulated 1.4 fold. In the two transposable element genes investigated (*TRANSPOSABLE ELEMENT1* and *SADHU1-1*), *afb2* is the only line in which both genes are up regulated, 2.7 and 3.3 fold, respectively. *afb3* is the only line to show increased expression of *UNKNOWN1* and *afb2* is the only line to show decreased expression of *UNKNOWN4*. All lines show upregulation of *LURP1*, with *afb1*, *afb2* and *afb3* upregulated 1.3 to 1.9 fold, *tir1* 4 fold, *pfl2* 16 fold and *pfl1* 179 fold. Several weak trends were identified, although no solid conclusions can be drawn. More r-protein and auxin mutants need to be investigated.

A major limitation of these experiments lies in the absence of replicates. Analysis with a single sample tends to return conservative results, therefore, possibly missing a number of genes with minor fluctuations in level of expression. This could be the case with miRNAs as small changes in expression levels can have dramatic effects on cellular transcriptional status as a result of plant miRNAs targeting both transcription factors and F-box proteins. By generating comprehensive transcriptomes from mutants within all three pathways we will be able to determine with much greater accuracy if an interplay does occur between auxin transcripts regulated by miRNAs with a dependency on efficient translation in Arabidopsis.

### **CHAPTER 3. USING NEXT GENERATION SEQUENCING TO ANALYZE *pfl1* (*rps18a*) AND *pfl2* (*rps13a*) RIBOSOMAL PROTEIN MUTANTS – Relationship to the thesis in its entirety**

Chapter 3 relates to the thesis as it takes a global approach in determining the genes responsible for generating the *pointed first leaf* phenotype in the *pfl1* (*rps18a*) and *pfl2* (*rps13a*) r-protein mutants by whole transcriptome analysis. Results obtained from the r-protein mutants were then compared to four auxin mutants, which display similar phenotypes, in an attempt to link ribosome biogenesis to auxin synthesis and transport through micro RNAs and thus link the three processes in plant development.

## CHAPTER 4. THE *rps18a:HF/RPL18B* DOUBLE MUTANT GENERATES A NOVEL PHENOTYPE

Mutating individual *Arabidopsis* r-proteins can result in lethality at the embryo stage, pointed first rosette leaves or no phenotype. Double mutants within the same r-protein gene family typically result in lethality. A r-protein mutation accompanying a mutation in genes involved in leaf dorsoventral patterning results in exaggerated leaf phenotypes. Embryo lethality due to a r-protein mutation has been attributed to a decreased overall translational efficiency; cells are unable to generate the required proteins necessary to proceed past the globular embryo stage. Degenhardt and Bonham-Smith (2008) have proposed that the pointed first leaf phenotype may result in translation dependent miRNA degradation of several auxin response factors (ARFs), therefore, still attributing the phenotype to a decrease in translational efficiency. Here we document a novel r-protein double mutant phenotype. The *pfl1(rps18a):HF/RPL18B* F<sub>1</sub> heterozygote exhibits a late flowering phenotype accompanied by an increased number of rosette leaves prior to bolting, increased primary bolt height and diameter, large, dark green, curled and highly serrate leaves and an extended life cycle. Whereas the *pfl2(rps13a):HF/RPL18B* F<sub>1</sub> heterozygous plant restore WT morphology and development. From these observations, I propose a unique extraribosomal function for RPS18A specific to vegetative development.

### 4.1 Introduction

All organisms require a strictly regulated system of producing proteins. This process is accomplished by ribosomes, massive 2.5 – 4.5 MD ribonucleoprotein particles (RNP). Ribosomes are composed of one large and one small subunit, both comprised of ribosomal RNA (rRNA) and ribosomal proteins (r-proteins). In addition to their structural roles in the ribosome, r-proteins may also function in mRNA binding, movement of the nascent polypeptide through the exit tunnel, monitoring the ribosomal state, as well as, a variety of extraribosomal functions (Brodersen and Nissen, 2005).

*Arabidopsis* r-protein mutants can display a variety of phenotypes ranging from embryo lethality with the loss of function of a dominant paralog to no visible phenotype when a low expressing gene family member is mutated (Byrne, 2009). Both LSU (e.g *RPL2*, *RPL8*, *RPL23*, *RPL19* and *RPL40*) and SSU (e.g *RPS6* and *RPS11*) r-protein mutants can result

in embryo lethality at the globular embryo stage due to the loss of function of a dominant paralog (Tzafrir et al., 2003; Tzafrir et al., 2004; Meinke et al., 2008; Byrne, 2009).

However, it is the *pointed first leaf* phenotype that may yield the most insight into cellular processes and the effects of abnormal ribosome biogenesis on these processes. Mutations in *RPS18A*, *RPS13A*, *RPL5A*, *RPL5B*, *RPL9C*, *RPL10aB*, *RPL23aA*, *RPL24B* and *RPL28A* all result in narrow pointed first leaves, aberrant leaf venation, increased marginal serrations, reduction in palisade mesophyll cells and reduced cellular division (Vanlijsebettens et al., 1994; Ito et al., 2000; Nishimura et al., 2005; Degenhardt and Bonham-Smith, 2008; Pinon et al., 2008; Yao et al., 2008; Fujikura et al., 2009).

The *RPS18* and *RPS13* gene families are comprised of three and two alleles respectively. The three alleles of *RPS18* encode identical proteins while *RPS13* isoforms share 99% sequence similarity at the primary sequence level.

### ***RPS18A***

A T-DNA insertion in *RPS18A* resulted in the *pfl* phenotype characterized by pointed first rosette leaves, a decrease in the vegetative fresh weight, a reduction in root growth and an increased time to life cycle completion of approximately 10 days due to a delayed transition from vegetative to reproductive growth (Vanlijsebettens et al., 1991). The *RPS18A* promoter fused to the *GUS* reporter gene, showed highest expression in mitotically active tissues such as apical and lateral root meristems, shoot apical meristems (SAM), leaf primordial and floral meristems with exceptionally high activity in heart stage embryos (Vanlijsebettens et al., 1994). High embryonic r-protein gene expression in plants correlates with that of r-protein gene expression in *Drosophila*. Several *Drosophila* r-protein mutants die as embryos or at the first instar larva stage, highlighting the importance of these r-proteins during embryogenesis (Kay and Jacobslorena, 1987). *Drosophila minute* mutants result from mutations in any one of a number of cytosolic r-proteins including *RPS18* and *RPS13* (Saeboe-Larsen and Lambertsson, 1996). In addition to the high level of *RPS18A* activity in rapidly dividing cells, elevated levels of *RPS18A* have also been identified in tissues following mechanical stress. Enhancer-like repeats (similar to those found in wheat glutenin, pea legumin (Shirsat et al., 1989), human interferon inducible gene 6-16 (Porter et al., 1988) and the CaMV 35S promoter (Fang et al., 1989))

located in the promoter and 3' end suggest that *RPS18A* may be regulated by an array of mechanisms in response to a variety of stimuli (Vanlijsebettens et al., 1994).

RPS18 appears to be highly conserved between eukaryotes with 74% amino acid sequence identity among *Arabidopsis* and rat (Chan et al., 1991), mouse and human RPS18 (Macmurray and Shin, 1992; Chassin et al., 1993). While a functional characterization of plant RPS18 has yet to be carried out the prokaryotic homolog RPS13 has been characterized in *E. coli*, *Bacillus stearothermophilus* and *B. subtilis* (Chan et al., 1991; Macmurray and Shin, 1992; Chassin et al., 1993). RPS13 interacts with initiation factors, 16S rRNA, (Lake, 1985; Moore and Capel, 1988; Lindahl and Zengel, 1991) and in conjunction with RPS12 interacts with control elements for the translocation of the mRNA:tRNA complex during translation (Cukras et al., 2003). Due to the high sequence similarity between the *Arabidopsis* RPS18 and prokaryotic RPS13, we suggest that RPS18 will have a similar role in translation.

### ***RPS13A***

In *Arabidopsis*, disruption of *RPS13A* by a Ds transposon insertion resulted in the *pfl2* r-protein mutant. As with *pfl1*, *pfl2* exhibits a late transition from vegetative to reproductive growth of approximately one week, therefore, prolonging its life cycle, as well as pointed first leaves (Ito et al., 2000). Phenotypes unique to the *pfl2* mutant include root growth inhibition and abnormal trichome morphology (Ito et al., 2000). As with *RPS18A* and most other r-proteins, *RPS13A* has its highest expression levels in mitotically active tissues such as the SAM, young leaves, young hypocotyls, axillary buds and young flower buds (Vanlijsebettens et al., 1994; Williams and Sussex, 1995). The high level of r-protein expression most likely correlates to the increased protein synthesis requirements of cells undergoing rapid cellular growth and division.

*Arabidopsis* RPS13A shares 79 – 92% amino acid sequence similarity to pea, maize (Joanin et al., 1993), humus earthworm, rat (Suzuki et al., 1990) and human (Chadeneau et al., 1993) RPS13 proteins suggesting that function along with structure is most likely conserved. In rat, RPS13 along with several other r-proteins forms a ribonucleoprotein complex with 5.8S rRNA, which binds ternary complexes of eIF-2:Met-tRNA:GTP and EF-1 $\alpha$ :Phe-tRNA:GTP (Chan et al., 1982). This association suggests that RPS13 has an active

role in translation as part of the amino acyl-tRNA binding domain in the ribosome (Chan et al., 1982). Ito et al., (2000) have suggested another role for Arabidopsis RPS13 in trichome development. Trichome morphology in *pfl2* resembles that of *glabra1* (*gl1*) and *glabra3* (*gl3*) mutants with mutations in trichome cell morphogenesis and the initiation of endoreplication, suggesting that RPS13 may interact directly or indirectly with GL1 or GL3 or may be involved in some other aspect of trichome development (Folkers et al., 1997; Hulskamp et al., 1998).

### **35S:HF/RPL18B**

A 35S:HF/RPL18B overexpressor in Arabidopsis was originally designed as a means to immunopurify polysome complexes (Zanetti et al., 2005). Based on 50S subunit structural data from *Haloarcula marismortui* it was believed that the addition of a six His residue tag and a FLAG epitope to the amino terminus of RPL18B would result in both tags being exposed on the solvent side of the ribosome therefore making immunopurification of ribosomes possible (Zanetti et al., 2005). Using an anti-FLAG antibody it was shown that HF/RPL18B was efficiently incorporated into ribosomes, with no noticeable effect on plant development (Zanetti et al., 2005). HF/RPL18B was also successfully located in LSU, monosome and polysome fractions but not free mRNA or SSU fractions further confirming the functionality of the HF/RPL18B protein during a normal lifespan of these plants when no environmental or developmental stresses are present (Zanetti et al., 2005).

Several r-protein double mutants have been produced in Arabidopsis; (1) multiple members of the same r-protein family, (2) r-proteins in different families and (3) a single r-protein in conjunction with a second gene with unrelated ribosome function. The homozygous *pfl1/pfl2* double mutant resembled both *pfl1* and *pfl2* single mutants when grown at 22°C and more closely resembled the *pfl2* single mutant when grown at 13°C (a decreased temperature exaggerated the *pfl2* phenotype) (Ito et al., 2000). These results suggest that *rps18a* (*pfl1*) and *rps13a* (*pfl2*) are independently involved in common developmental pathways as a more severe phenotype was not produced in the double mutant (Ito et al., 2000). When multiple members of a single r-protein gene family are knocked down, the results can be dramatic. In the two-member gene family *RPL23aA/B*,

RNAi mediated knock down of both genes was lethal (Degenhardt and Bonham-Smith, 2008). This outcome was also seen when all three cytosolic members or the two non-cytosolic members of the *RPS15a* gene family are knocked down by RNAi, indicating that members from both sub-families are absolutely required during early plant development (Chapter 2). The *piggyback 1* (*pgy1*), *pgy2*, *pgy3* (*rpl10ab*, *rpl9c* and *rpl5a*, respectively) single mutants display a *pfl* phenotype (less severe than that found in the *pfl1* and *pfl2* single mutants) and prominent marginal serrations, however, when these mutations were incorporated into an *asymmetric leaves1* (*as1*: phenotypes range from small patches of abaxial cells on the adaxial side to complete loss of adaxial cell fate (Tattersall et al., 2005; Pinon et al., 2008)) background, a severe *pfl* phenotype resulted with the generation of adaxial ectopic lamina (Pinon et al., 2008). RPL10aB, RPL9C and RPL5A contribute to adaxial leaf identity in a similar manner, as the *as1/pgy1/pgy2/pgy3* quadruple mutant showed little additional adaxial ectopic laminae compared to that of the individual *pgy/asa1* double mutants (Pinon et al., 2008). *PGY1* (*RPL10aB*), *PGY2* (*RPL9C*) and *PGY3* (*RPL5A*) affect dorsoventral patterning through interactions with the HD-ZIPIII-KANADI pathway (Pinon et al., 2008). Double mutants *asymmetric leaves1/2 enhancer5* (*ae5*) *rpl28a*:*as1/2*, *rpl5a* (*ae6*):*as1/2*, *rpl5b*:*as1/2* or *rpl24b*:*as1/2* all produce similar phenotypes to the *piggyback:as1* mutants thus they most likely function in a similar manner, through the HD-ZIPIII-KANADI pathway to regulate adaxial/abaxial leaf identity (Yao et al., 2008).

Here I present data demonstrating a novel r-protein double mutant phenotype resulting from crossing the *pfl1* (*rps18a*) and *HF/RPL18B* single mutants. The late flowering phenotype and enlarged primary bolt of the resulting double mutant suggests that RPS18A has a unique extraribosomal function during vegetative growth.

## 4.2 Material and methods

### 4.2.1. Plant material and growth conditions

Homozygous *Arabidopsis thaliana* mutants of *pfl1*, *pfl2* and *HF/RPL18B* were obtained from Arabidopsis Biological Resource Center (ARBC), Rikagaku Kenkyūjo (RIKEN) and Dr. Julia Bailey Serres (UC Riverside), respectively. All plants were grown under a 23°C/17°C temperature regime and 16/8 (light/dark) hour photoperiod of ~120 μmol

photons  $\text{m}^{-2} \text{s}^{-1}$ . Prior to pollen development sepals, petals and stamens were removed from immature flowers leaving the exposed stigma. Reciprocal crosses of each *pfl* mutant with *HF/RPL18B* were carried out. Resulting  $F_1$  seed was stratified for two days at  $4^\circ\text{C}$  and selected on  $\frac{1}{2}$  MS (2.17 g/L MS salts [PhytoTechnology Laboratories], 1.5% sucrose, 0.8% phytagar [Invitrogen], pH 5.7) kanamycin (50  $\mu\text{g}/\text{mL}$ ) plates and  $F_1$  plants were transferred to soil 14 days after plating.

For transient expression experiments, tobacco (*Nicotiana tabacum*) cultivar Petit Havana plants were grown under a  $23^\circ/18^\circ\text{C}$  temperature regime and a 16/8 (light/dark) hour photoperiod of  $\sim 170 \mu\text{mol photons m}^{-2} \text{sec}^{-1}$ . Young leaves from four to six-week-old plants were used for all infiltration experiments.

#### **4.2.2. Fluorescent protein constructs**

A heterologous system was used to determine the subcellular localization of each isoform encoded by the three gene families, *RPS18A/B/C*, *RPS13A/B* and *RPL18B/C*. Constructs were built as previously described in Section 2.2.5.

#### **4.2.3. Transient expression in tobacco and confocal microscopy**

Transient expression was performed as previously described in Section 2.2.7.

#### **4.2.4. Genevestigator transcript expression profiling**

Genevestigator ([www.genevestigator.com](http://www.genevestigator.com), 2011) was used to determine developmental stage specific mRNA expression patterns for all *RPS18*, *RPS13* and *RPL18* gene family members. Expression values for each developmental stage were obtained from ATH1:22K high quality arrays of wild type Arabidopsis, Columbia-0 (Hruz et al., 2008).

#### **4.2.5. Light microscopy**

Scape segments were taken approximately 25mm from the base of both WT and *pfl1:HF/RPL18B* plants. Samples were fixed in 2% gluteraldehyde for 48 hours, washed three times in a 25 mM  $\text{NaPO}_4$  buffer (five minutes per wash) and three times in 50% ethanol (five minutes per wash) before passing through a n-butanol dehydration series (50 mL  $\text{H}_2\text{O}$ : 40 mL EtOH: 10 mL n-butanol, 30:50:20, 15:50:35, 45 mL EtOH: 55 mL n-butanol,

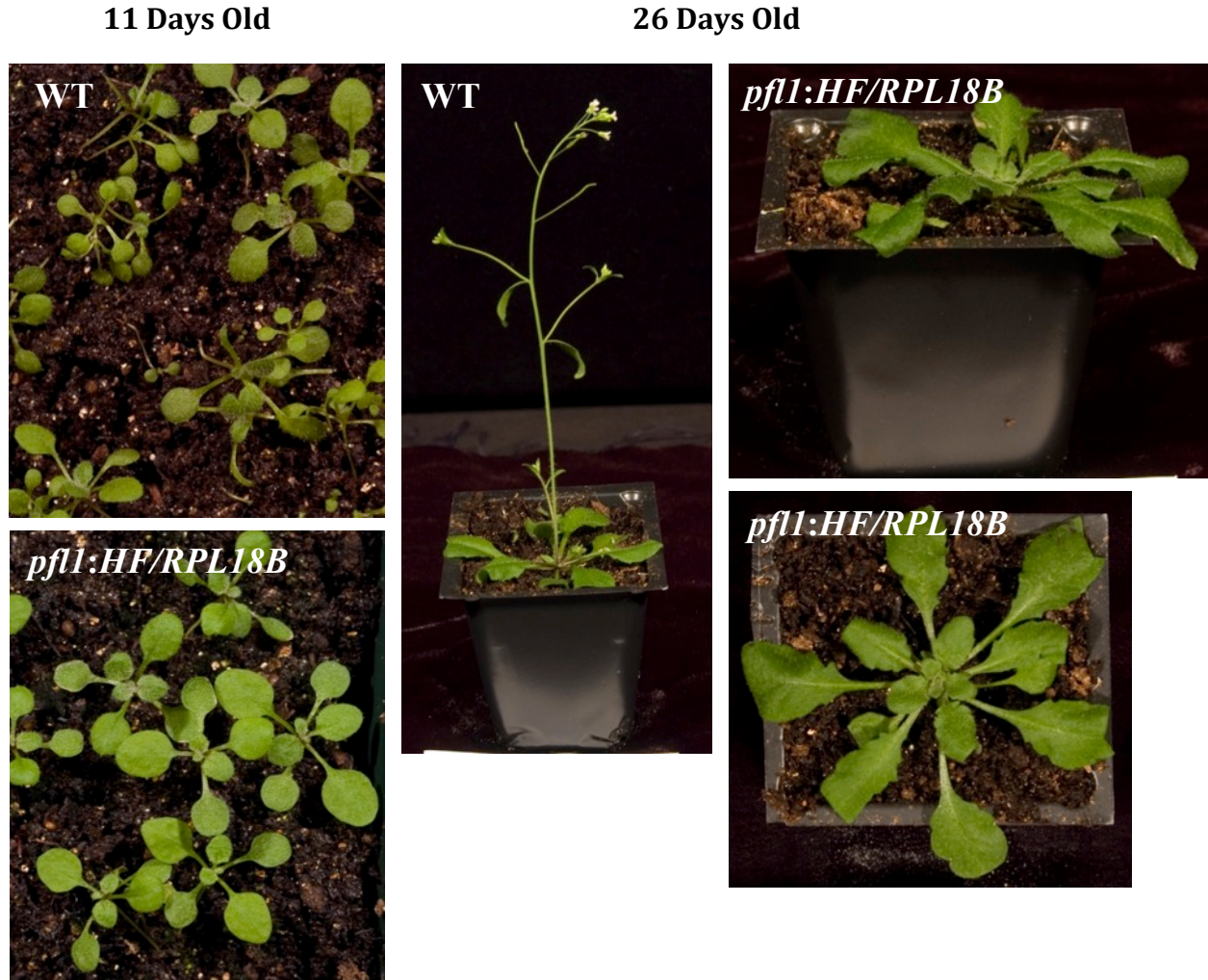


25:75, 100% n-butanol) each step for one hour with two additional 100% n-butanol steps (the last step overnight). Once fully dehydrated, samples were infiltrated with paraffin chips at 60°C, then embedded in 100% paraffin, mounted and sectioned. Sections were mounted and stained with toluidene blue for 10-15 minutes and any remaining paraffin was removed with sequential washes with xylene. Prepared sections were mounted in permount 24 hours before viewing with a light microscope.

## 4.3 Results

### 4.3.1. Heterozygous *pfl1:HF/RPL18B* double mutants result in a novel phenotype

To investigate the effects of a simultaneous reduction in a single r-protein and overproduction of a different r-protein have on plant development, two r-protein mutants were generated by crossing homozygous lines for each r-protein knockout mutant (*pfl1* (*pointed first leaf 1*: T-DNA insertion in *RPS18A*) and *pfl2* (*pointed first leaf 2*: Ds transposon insertion in *RPS13A*)) with a homozygous overexpressing *RPL18B*-His/FLAG (*HF/RPL18B*) tagged line. F<sub>1</sub> seedlings were selected on kanamycin (*pfl1*) or kanamycin/hygromycin (*pfl2*) plates. F<sub>1</sub> plants heterozygous for the *pfl2* (*rps13a*) and *HF/RPL18B* loci exhibited wildtype (WT) development and morphology while the *pfl1* (*rps18a*):*HF/RPL18B* F<sub>1</sub> heterozygotes displayed both aberrant development and morphology. Up to 21 days old both WT and double mutant plants were indistinguishable from each other (Figure 4.1); however, in the following 1-2 days WT plants began to bolt while the *pfl1:HF/RPL18B* double mutants were delayed (Figure 4.1). Whereas WT plants produced 9-10 rosette leaves prior to bolting, the *pfl1:HF/RPL18B* F<sub>1</sub> mutants produced between 26 – 37 dark green, curled leaves (Figure 4.2). Unique to the double mutant was the presence of a thickened bolt and a reduction in the number of secondary bolts. Double mutant primary bolts were 2.6 fold thicker than WT (Figure 4.3). Although all double mutant plants exhibited a delay in transition to flowering, they varied in terms of time required for bolting, ranging from 33 – 44 days post-sowing (Figure 4.2). While WT plants were in advanced stages of senescence after 54 days, the F<sub>1</sub> heterozygous double mutant plants were still in various stages of inflorescence development (Figure 4.4). *pfl1:HF/RPL18B* primary bolts were 21% taller than WT prior to senescence (Figure 4.5). Leaf vasculature,



**Figure 4.1. WT and *pfl1:HF/RPL18B* F<sub>1</sub> heterozygous double mutant up to 26 days.** At 11 days old both WT and the double mutant are indistinguishable from each other. This persists until 21 – 24 days, at which point WT plants begin to bolt while the double mutants begin to exhibit a late flowering phenotype. This is illustrated at 26 days old where the WT has a developed primary bolt and the double mutant has not.

39 Days Old

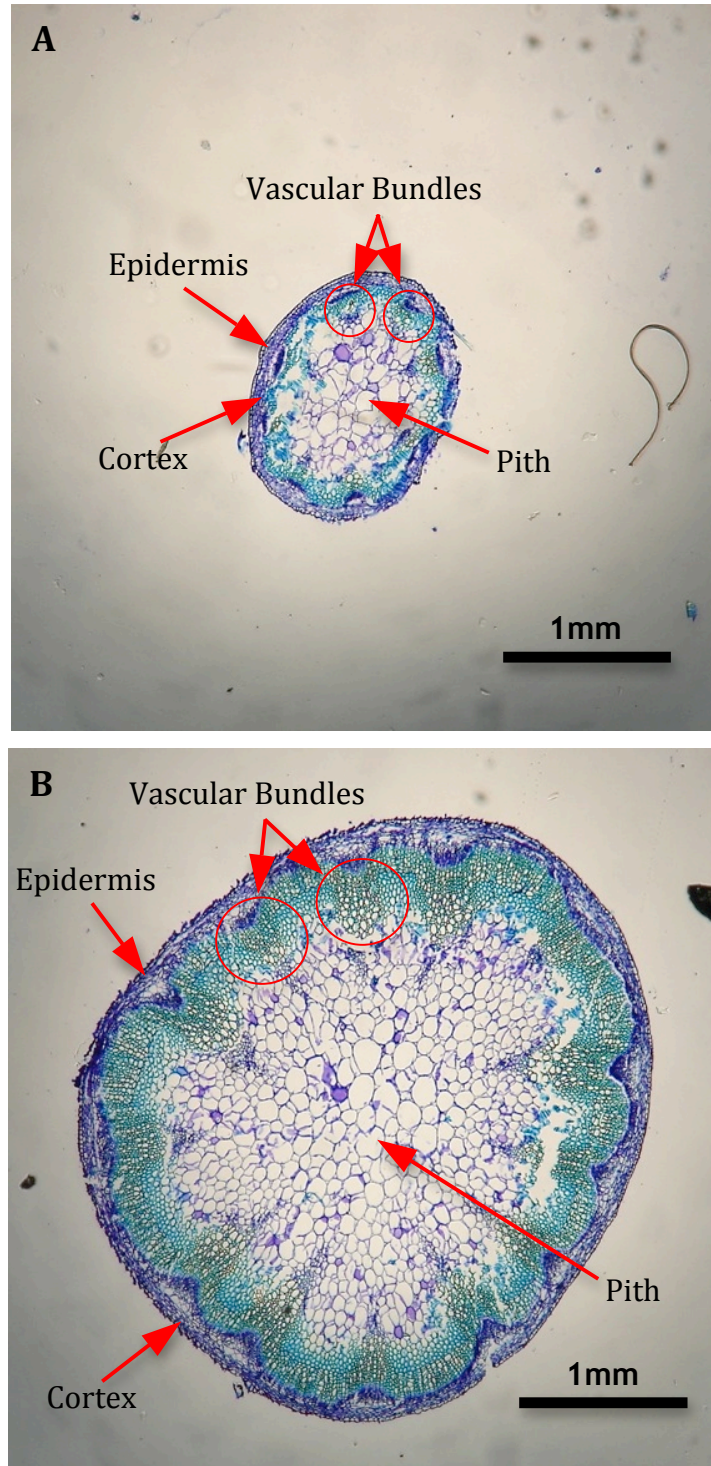


43 Days Old



**Figure 4.2. WT and *pfl1:HF/RPL18B* F<sub>1</sub> heterozygous double mutants up to 43 days.** Double mutants at 39 days old exhibit 26-37 dark green, curled and highly serrated leaves prior to bolting as compared to WT. At 43 days, WT plants are setting seed while double mutants have just begun the transition from vegetative to reproductive growth or are in the early stages of bolting.





**Figure 4.3. WT (A) and *pfl1/HF:RPL18B F1* (B) heterozygous double mutant primary bolt cross sections.** Sections were taken approximately 25mm from the base of the bolt prior to senescence and stained with Toluidine blue. (B) Double mutants had a 2.6 fold increase in diameter of the primary bolt compared to WT while basic bolt architecture remained very similar between the two. The double mutant contained more vascular bundles and an increased number of cells per bundle as well as more cells in the pith.

54 Days Old



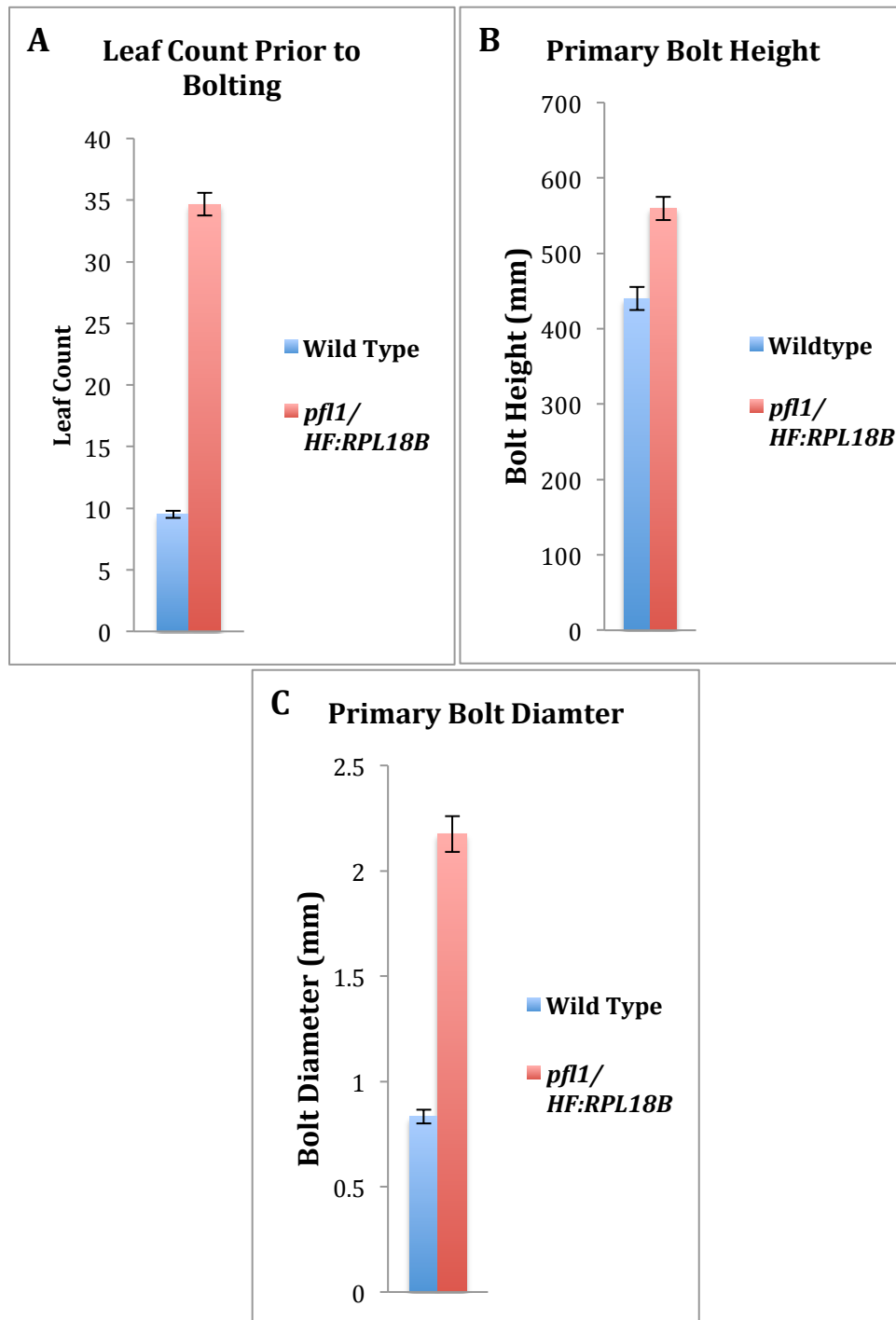
**Figure 4.4. WT and *pfl1:HF/RPL18B* F<sub>1</sub> heterozygous double mutant at 54 days.** WT plants are in advanced stages of senescence while the double mutants show a variety of late flowering phenotypes. The more delayed the transition from vegetative to reproductive growth the greater the number of leaves produced prior to bolting.

trichome morphology and root structure are all unaffected in *pfl1:HF/RPL18B* plants. It is important to note the difference between the delay in development exhibited by the *pfl1:HF/RPL18B* mutant and the delay in growth found in many single r-protein mutants. The delay observed in the double mutant was in the transition from vegetative to reproductive growth, increased flowering with a concomitant increase in the vegetative mass of the plant, while numerous single r-protein mutants exhibit a similar phenotype to that of the *Drosophila minute* phenotype, presenting a reduced growth overall, both in the timing of transition from vegetative to reproductive growth and a reduction in the mass of the plant.

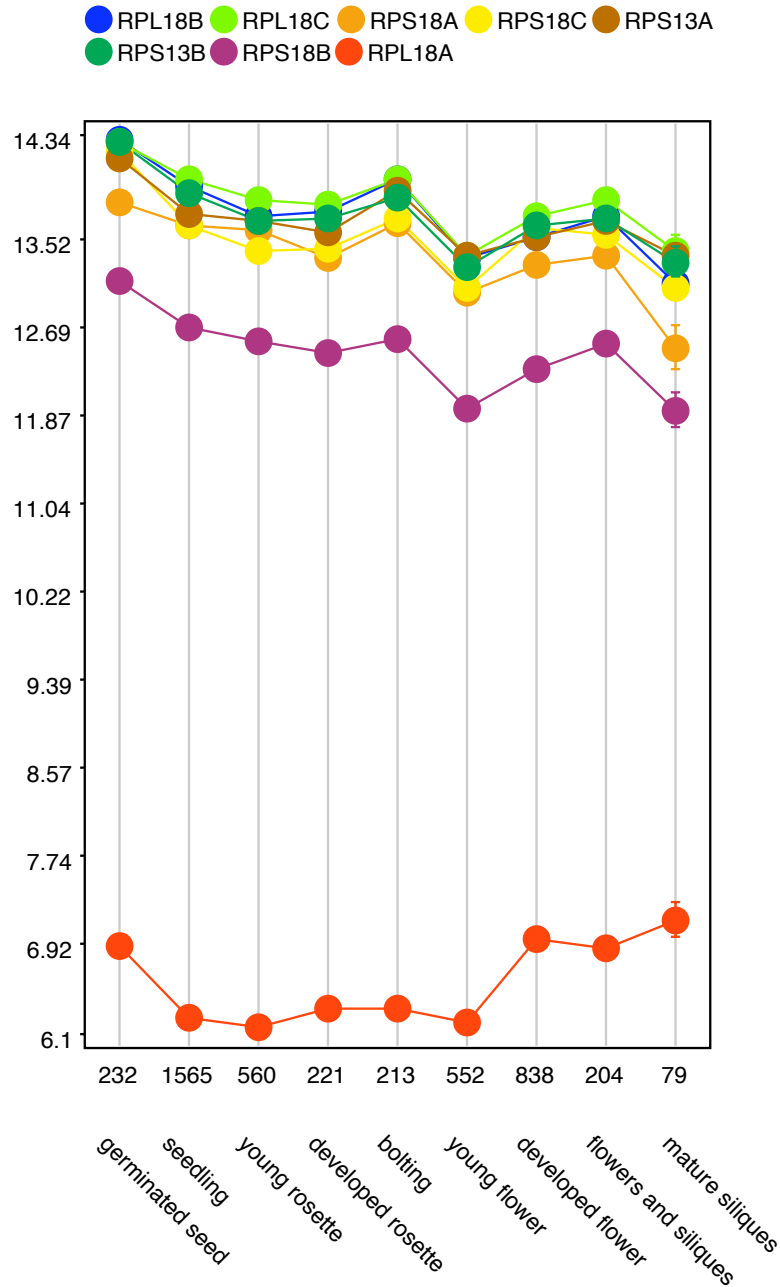
Prior to senescence, scapes, within 25mm of the base, were sampled from both WT and *pfl1:HF/RPL18B* plants (Figure 4.3). In the double mutant, there was an increase in number of pith cells and vascular bundles, presumably to compensate for the increase in primary bolt diameter. While the cell number increased in both vascular bundles and the pith of the double mutant, cell size did not.

#### **4.3.2. Transcript expression profiles of each member of the *RPS18*, *RPS13* and *RPL18* families are consistent and similar across a variety of developmental stages**

Genevestigator ([www.genevestigator.com](http://www.genevestigator.com), 2011) was used to determine developmental stage specific mRNA profiles for each gene family member. With the exception of *RPL18A*, the remaining r-protein genes (*RPS18A/B/C*, *RPL18B/C* and *RPS13A/B*) all showed similar expression levels and trends across the developmental stages examined (Figure 4.6). *RPL18A* was expressed at very low levels in all tissues and in contrast to the other r-proteins, showed its highest level of expression in the mitotically inactive tissues, developed flowers, flowers, siliques and mature siliques. This pattern is in contrast to most r-protein expression profiles where tissues with a high mitotic index have a higher r-protein transcript abundance. This unusual expression profile could be due to an as yet unidentified extraribosomal function for *RPL18A*, however, as I was unable to generate a *RPL18A* cDNA from a variety of RNA samples representing a number of developmental stages the expression levels recorded for *RPL18A* may have resulted from non-specific primer binding during microarray sample hybridization.



**Figure 4.5. WT and *pfl1:HF/RPL18B* F<sub>1</sub> heterozygous double mutant (A) leaf count prior to bolting, (B) bolt diameter and (C) bolt height measurements.** Double mutants had a 3.2 fold increase in the number of leaves produced prior to bolting, grew 21% taller than WT and had a 2.6 fold increase in the diameter of the primary bolt. WT, n=4, Double mutants, n=12.



**Figure 4.6. Development stage specific mRNA expression profiles of the *RPS18*, *RPS13* and *RPL18* gene families.** *RPS18A/ B/ C*, *RPS13A/ B* and *RPL18B* and *C* all exhibit similar transcript levels with respect to each other in each developmental stage, as well as, similar levels of relative expression over these developmental stages. *RPL18A* shows a weak presence in all developmental stages but has its highest transcript level in mitotically inactive tissues. Y – axis – Level of expression (signal intensity on ATH1: 22K high quality array). X – axis – developmental stage. n = 232 (germinated seedling), n = 1565 (seedling), n = 560 (young rosette), n = 221 (developed rosette), n = 213 (bolting), n = 552 (young flower), n = 838 (developed flower), n = 204 (flowers and siliques) and n = 79 (mature siliques). Analysis through Genevestigator (<https://www.genevestigator.com/gv/>).



### 4.3.3. Nuclear and nucleolar subcellular localization of RPS13, RPS18 and RPL18 proteins.

The three members of the *RPS18* gene family (*RPS18A/B/C*: T-DNA insertion in *RPS18A* yields the *pfl1* mutant) have 87% nucleotide sequence identity among the CDSs and 100% amino acid identity (Figure 4.7). All three proteins localized to the nucleus and nucleolus with nucleolar localization most prominent (Figure 4.8). However, RPS18C localized to the nucleus, with a lower intensity than RPS18A/B and appeared more intense at the periphery of the nucleus (Figure 4.8J-L). These results are surprising as all three cDNAs encode identical proteins. All proteins also produced a strong cytoplasmic signal, most likely an artifact of using the 35S promoter in pGREEN (Figure 4.8M).

#### ***RPS13* Family**

*RPS13* has two family members, *RPS13A/B* (Ds transposon insertion in *RPS13A* produces the *pfl2* mutant) sharing 84 and 99% in the CDSs and polypeptide sequences, respectively (Figure 4.7). Both proteins exhibit moderate nuclear and strong nucleolar localization (Figure 4.9D-I). A cytoplasmic signal is also evident but less intense than that for RPS18A/B/C (Figure 4.9 and 4.8M).

#### ***RPL18* Family**

*RPL18A/B/C* comprise the three members of the *RPL18* gene family. *RPL18B/C* CDSs share 89% identity but that drops to 74 – 75% when each are compared to *RPL18A*. *RPL18A* is 53 amino acids shorter than *RPL18B/C* and shows 77% amino acid identity to *RPL18B/C* while *RPL18B/C* are 95% similar to each other (Figure 4.7). Similar to the RPS18 and RPS13 isoforms, *RPL18B/C* exhibits strong nucleolar and moderate nuclear localization (Figure 4.9J-O) but has a weak cytoplasmic signal more reminiscent of the RPS13 isoforms (Figure 4.9N). *RPL18A* was unavailable from ABRC (July 2012) nor could a transcript suitable for cloning be generated by RT-PCR from seedling or mature plant tissues. The inability to isolate a *RPL18A* cDNA correlates with the weak expression pattern found in Genevestigator (Figure 4.6) suggesting that expression of this transcript may be environmental/developmental/tissue dependent and only very low expression is required

### **RPS18**

```
RPS18A MSLVANEEFQHILRVLNTNVDGKQKIMFALTSIKGIGRRLANIVCKKADVDMNKRAGELS 60
RPS18B MSLVANEEFQHILRVLNTNVDGKQKIMFALTSIKGIGRRLANIVCKKADVDMNKRAGELS 60
RPS18C MSLVANEEFQHILRVLNTNVDGKQKIMFALTSIKGIGRRLANIVCKKADVDMNKRAGELS 60
*****

RPS18A AAEIDNLMITIVANPRQFKIPDWFLNRQKDYKDGKYSQVVSNALDMKLRDDLERLKKIRNH 120
RPS18B AAEIDNLMITIVANPRQFKIPDWFLNRQKDYKDGKYSQVVSNALDMKLRDDLERLKKIRNH 120
RPS18C AAEIDNLMITIVANPRQFKIPDWFLNRQKDYKDGKYSQVVSNALDMKLRDDLERLKKIRNH 120
*****

RPS18A RGLRHYWGLRVRGQHTKTTGRRGKTVGVSKKR 152
RPS18B RGLRHYWGLRVRGQHTKTTGRRGKTVGVSKKR 152
RPS18C RGLRHYWGLRVRGQHTKTTGRRGKTVGVSKKR 152
*****
```

### **RPS13**

```
RPS13A MGRMHSRGKGISASALPYKRSSPSWLKTTTPQDVDESICKFAKKGLTPSQIGVILRDSHGI 60
RPS13B MGRMHSRGKGISASALPYKRSSPSWLKTTSDQDVDESICKFAKKGLTPSQIGVILRDSHGI 60
*****

RPS13A PQVKSVTGSKILRILKAHGLAPEIPEDLYHLIKKAVAIRKHLERNRKDKDSKFRLILVES 120
RPS13B PQVKSVTGSKILRILKAHGLAPEIPEDLYHLIKKAVAIRKHLERNRKDKDSKFRLILVES 120
*****

RPS13A RIHRLARYYKKTKKLPPVWKYESTTASTLVA 151
RPS13B RIHRLARYYKKTKKLPPVWKYESTTASTLVA 151
*****
```

### **RPL18**

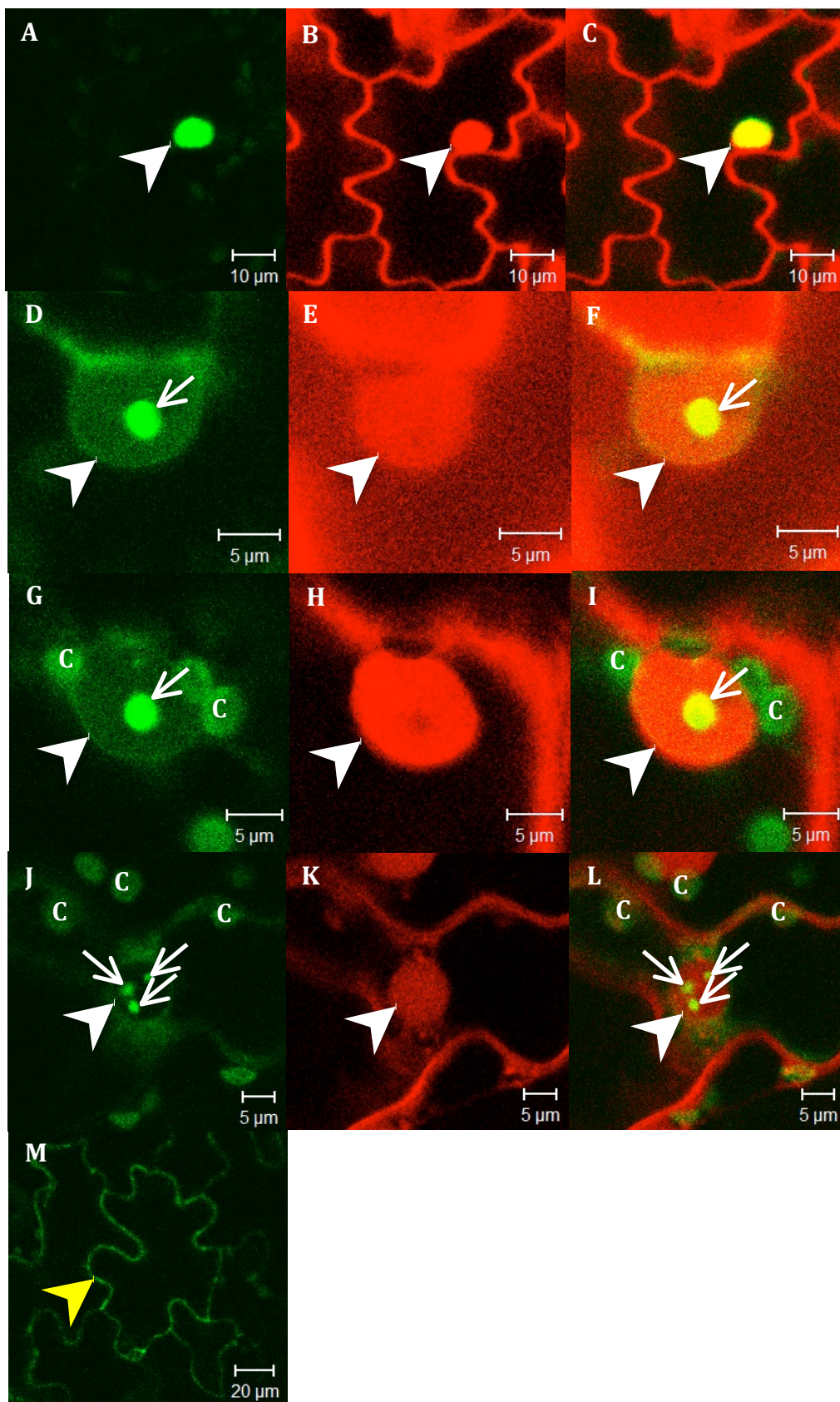
```
RPL18A -----MSKVNKA 7
RPL18B MGIDLIAGGKSKKTKRTAPKSDDVYLKLTVKLYRFLVRRINSKFNQVILKRLFMSKVNKA 60
RPL18C MGIDLIAGGKSKKTKRTAPKSDDVYLKLLVKLYRFLVRRSNSNFNAVILKRLFMSKVNKA 60
*****

RPL18A PLSLSRLVRYMDGKDGIKIAVIVGTVTDDVRIEDVPALTVTALRFTESARARIHKAGGECL 67
RPL18B PLSLSRLVEFMTGKEDKIAVLVGTITDDLRLVHEIPAMKVLTALRFTERARARIEKAGGECL 120
RPL18C PLSLSRLVEFMTGKDDKIAVLVGTITDDLRLVHEIPAMKVLTALRFTERARARIEKAGGECL 120
*****

RPL18A TFDQLALPCPTWSENTVLLRGPKNTREAVKHFGPAPGVPHSHTKPYVRQTGKKIEIARGR 127
RPL18B TFDQLALRAP-LGQNTVLLRGPKNSREAVKHFGPAPGVPHSHSKPYVRAKGRKFEKARGK 179
RPL18C TFDQLALRAP-LGQNTVLLRGPKNSREAVKHFGPAPGVPHSNTKPYVRHKGRKFEKARGK 179
*****

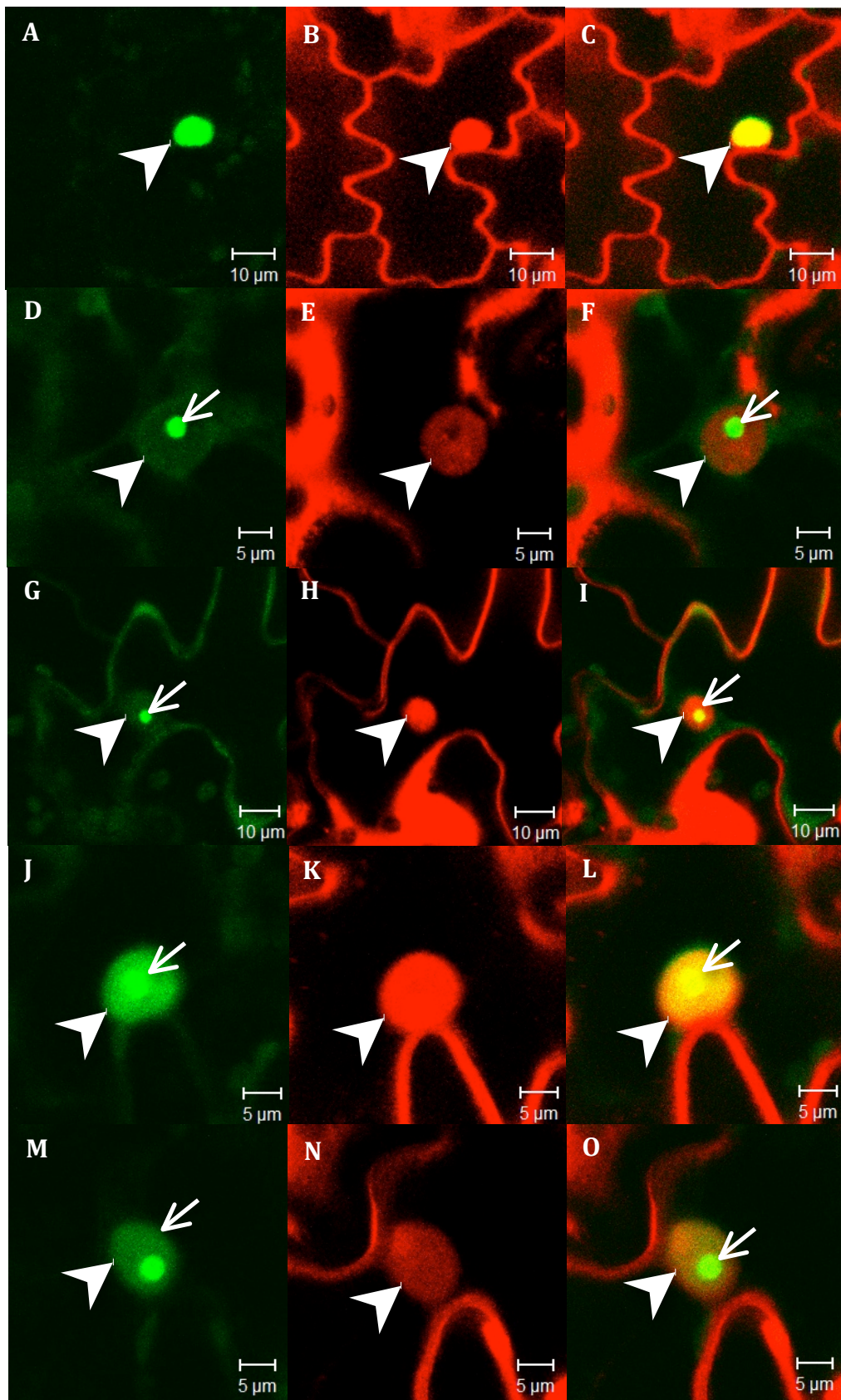
RPL18A RRSRGFKV 135
RPL18B RKSRGFKV 187
RPL18C RKSRGFKV 187
*****
```

**Figure 4.7. Amino acid alignment of the proteins encoded by the *RPS18*, *RPS13* and *RPL18* gene families.** *RPS18A*, *B* and *C* encode 100% identical proteins; *RPS13A* and *B* encode proteins that are 99% similar. RPL18A contains 53 fewer amino acids than RPL18B and C and shows 77% polypeptide identity to RPL18B and C while RPL18B and C are 95% similar to each other. Amino acids highlighted in yellow are putative NLSs as determined by the NLS prediction software NLStradamus (Ba et al., 2009). Pink highlighted amino acids differ between RPL18B and C in the first 53 amino acids.



**Figure 4.8. Cellular localization of the proteins encoded by the *RPS18* gene family.** (A, D, G, J) GFP images, (B, E, H, K) DAPI images and (C, F, I, L) GFP and DAPI images merged. (A-C) Nuclear localization of FIBRILLARIN. (D-F) Nuclear and nucleolar localization of RPS18A. (G-I) Nuclear and nucleolar localization of RPS18B. (J-L) Nuclear and nucleolar localization of RPS18C. (M) All proteins exhibited varying degrees of cytoplasmic localization. White arrows indicate the nucleolus, white arrowheads indicate the nucleus, yellow arrowheads indicate a strong cytoplasmic signal.





**Figure 4.9. Cellular localization of proteins encoded by the *RPS13* and *RPL18* gene families.** (A,D,G,J,M) GFP images, (B,E,H,K,N) DAPI images and (C,F,I,L,O) merged images. (A-C) Fibrillarin nuclear localization. (D-F) Nuclear and nucleolar localization of RPS13A. (G-I) Nuclear and nucleolar localization of RPS13B. (J-L) Nuclear and nucleolar localization of RPL18B. (M-O) Nuclear and nucleolar localization of RPL18C. White arrows indicate the nucleolus, white arrowheads indicate the nucleus.

for its role in flower senescence and silique development. However, low levels of expression may also be indicative of *RPL18A* as an inactive pseudogene.

#### 4.4 Discussion

The *pfl1:HF/RPL18B* F<sub>1</sub> heterozygous double mutant exhibited a late flowering phenotype, with the onset of bolting beginning anywhere from 33-44 days post-sowing compared to 21-24 days for WT. This late flowering phenotype is similar to that seen in a variety of Arabidopsis late flowering mutants in three of the four major flowering pathways. The four prominent flowering pathways are separated based on photoperiod, autonomous, vernalization or gibberellin regulation (Blazquez et al., 2001; Lim et al., 2004).

Phytochromes and cryptochromes monitor environmental light signals and relay information to circadian clock machinery, therefore, regulating time to flowering through photoperiod (Blazquez et al., 2001; Lim et al., 2004). The autonomous pathway involves a number of genes encoding proteins that suppress *FLOWERING LOCUS C* (*FLC*: encodes a MADS box transcription factor) (Michaels and Amasino, 1999). *FLC* expression, which promotes late flowering, is inhibited by three RNA binding proteins, *FCA*, *FPA* and *FLOWERING LOCUS K* (*FLK*) (Mockler et al., 2004; Feng et al., 2011), two chromatin remodeling proteins, *FVE* and *FLOWERING LOCUS D* (*FLD*) (Ausin et al., 2004; Kim et al., 2004), *LUMINIDEPENDENS* (*LD*: putative transcription factor that contains a divergent homeodomain) (Lee et al., 1994) and a homolog of yeast polyadenylation factor Pfs2p (*FY*) (Macknight et al., 1997; Schomburg et al., 2001; Simpson et al., 2003; Feng et al., 2011). In response to vernalization, *FRIGIDA* (*FRI*) and *FLC* are involved in regulating the transition to reproductive growth. In the absence of vernalization, *FRI* expression promotes *FLC* expression, which delays flowering. Following vernalization, *FRI* expression is down regulated resulting in decreased *FLC* expression and earlier flowering (Lim et al., 2004; Shindo et al., 2005). The fourth flowering pathway is regulated by gibberellin (GA), with *ga1* (*GA1* encodes copalyl diphosphate synthase that is required to catalyze the first step in GA synthesis) (Sun and Kamiya, 1994), *ga4* (*GA4* encodes GA 3B-hydroxylase) and *ga5* (*GA5* encodes GA 20-oxidase) (Talon et al., 1990) mutants all showing delayed flowering as a result of decreased GA biosynthesis (Jacobsen et al., 1996; Mouradov et al., 2002).

The delayed flowering phenotype of the *pfl1:HF/RPL18B* mutant may simply be the result of disrupted ribosome biogenesis. The concomitant reduction of *RPS18A* and overproduction of *RPL18B* may be a sufficient stress to decrease the quantity of functional ribosomes, possibly, altering the ratio of flowering promoting proteins (FCA, FPA, FVE, FLD, LD, FY, GA1, GA4 and GA5) to flowering delaying proteins (FRI, FLC, SPY, GAI, RGA and RGL1). A similar phenotype was not observed in the *pfl2:HF/RPL18B* double mutant, possibly due to gene compensation by *RPS13B*. Decreased transcript levels from *rps13a* may be compensated for by the presence of the functional *RPS13B*, resulting in no change in the ratio of functional to non-functional ribosomes. The *pfl1:HF/RPL18B* mutant phenotype suggests that this is not the case in the *RPS18* gene family where *RPS18B* and *RPS18C* are unable to compensate for the loss of *RPS18A*, resulting in a decreased pool of functional ribosomes in this mutant. The ribosome heterogeneity model proposed by Horiguchi et al., (2012) provides a possible mechanism for this scenario. This model assumes that multiple expressed members of each family have non-equivalent functions; therefore, ribosome specificity will vary with respect to the incorporated r-protein isoform. To overproduce RPL18B may specifically require RPS18A containing ribosomes, with no such specificity for RPS13A or B, resulting in the different phenotypes associated with the two heterozygous double mutants. Ribosome insufficiency may also be occurring in the *pfl1:HF/RPL18B* plants where *RPS18A* may be the dominant paralog and reduced expression would result in decreased numbers of fully functional ribosomes. Recently, *rps18c* was shown to exhibit a *pfl* phenotype similar to that of *rps18a* mutants (Horiguchi et al., 2011) suggesting redundant roles for the *RPS18* family members and that ribosome insufficiency is most likely not responsible for the observed mutant phenotype. To confirm this suggestion, *RPS18B* must be mutated and investigated for abnormal phenotypes.

Ribosome aberrancy may also be a contributing factor to the mutant phenotypes. This model assumes that all members of a family have equivalent functions within families but differ among different families (Horiguchi et al., 2012). This provides the simplest explanation as the concomitant overproduction of RPL18B and decreased production of RPS18A may result in aberrant ribosomes unable to translate a required subset of transcripts whereas the ribosomes in *rps13a:HF/RPL18B* plants are able to translate this same subset of transcripts. If we assume that the subset of transcripts is required for



normal leaf development, the ribosome aberrancy model explains the different phenotypes observed in the two double mutant plants.

When the *pfl1:HF/RPL18B* F<sub>1</sub> mutant started to bolt (starting anywhere from 12 – 31 days after WT) it produced a thickened primary inflorescence with a bolt approximately 2.6 fold larger in diameter than WT. Primary inflorescence thickening has previously been reported in *enhanced ethylene response1* (*EER1*: encodes a protein that acts to oppose ethylene responses) (Larsen and Chang, 2001), *constitutive ethylene-response* (*CTR*: encodes a negative regulator of ethylene responses) (Kieber, 1997; Larsen and Chang, 2001), *revoluta* (*REV*: encodes a class III homeodomain-leucine zipper protein that regulates interfascicular fiber differentiation and other apical meristems) (Talbert et al., 1995; Lev-Yadun et al., 2004) and *erecta* (*ER*: encodes a receptor protein kinase and is responsible for the Arabidopsis Landsberg *erecta* ecotype) (Torii et al., 1996) mutants. *eer1* and *ctr* plants both produce enlarged primary bolts under normal growing conditions as both are incapable of controlling endogenous ethylene levels, however, in both mutants, the number and size of rosette leaves, primary bolt height, flower morphology or timing of flowering, are not affected (Larsen and Chang, 2001).

Mutations in *REV*, which normally promote paraclade, floral and primary shoot meristem growth while inhibiting leaf meristem, floral organ and stem growth, result in numerous abnormal phenotypes including increased primary bolt diameter. Compared to WT, internodal bolt diameter is 18% larger in *rev* plants and plant height is reduced by 52%. Along with the enlarged bolts, large, irregular shaped, dark green rosette leaves are prominent in the *rev* plants. Unlike *pfl1/HF:RPL18B* plants, phenotype abnormalities in *rev* plants are not specific to vegetative tissue. Fertile flowers are enlarged and produce larger seeds than WT while sterile flowers are missing pistils and are smaller and abnormally shaped (Talbert et al., 1995).

Perhaps the most well documented case of primary bolt thickening is found in the Landsberg *erecta* ecotype of Arabidopsis (Hwang et al., 1991). The *erecta* phenotype associated with this mutant is due to a mutation in *ER* that is believed to be involved in an intercellular signaling pathway required for organ development from the SAM (Torii et al., 1996). As such, *er* phenotypes are more associated with reproductive as opposed to vegetative tissues. The most common phenotypes are clustering of flower buds at the top

of the primary bolt, short, wide siliques and decreased overall plant height (Torii et al., 1996).

None of these mutants adequately describe the enlarged primary bolt phenotype of the *pfl1:HF/RPL18B* mutant. However, it is apparent that multiple pathways can lead to thickened primary bolts. The *pfl1:HF/RPL18B* double mutant combines a late flowering phenotype with an enlarged primary bolt, the first time these two phenotypes have been linked in Arabidopsis. This novel combination suggests a unique extraribosomal role for RPS18A in linking the transition from vegetative to reproductive growth to primary bolt development.

Many r-proteins possess extraribosomal, in addition to ribosomal, functions. The ability of r-proteins to function outside their “normal” ribosomal environment could be attributed to their evolutionary origins. Two theories prevail: (1) r-proteins were selected specifically for their function in ribosomes or (2) proteins with previously defined functions were adopted and adapted to ribosomes, thereby, allowing ribosomes to function more efficiently in a variety of cellular environments (Wool, 1996). In *E. coli*, yeast, human, *Drosophila*, *Xenopus laevis*, rat and Arabidopsis r-proteins with functions independent of ribosome biogenesis or function have been identified, e.g. replication (*E. coli* RPS1 and RPL14), transcription (*E. coli* RPS10 and yeast RPS20), DNA repair (*E. coli* RPS9 and human and *Drosophila* RPS3), mRNA processing (*E. coli* RPS12: enhances phage T4 intron splicing), regulation of development (mutations in approximately 50 r-proteins can confer the *minute* phenotype in *Drosophila*), establishment of leaf adaxial/abaxial identity (RPL10a, RPL9, RPL5, RPL28A, RPL5A, RPL5B and RPL24B) and regulation of the cell cycle in mammals (RPL5, RPL7, RPL11, RPL23, RPL26, RPL29, RPL30, RPL37, RPS3, RPS6, RPS7, RPS9 and RPS27) (Chan et al., 1982; Wool, 1996; Horn and Vousden, 2008; Pinon et al., 2008; Yao et al., 2008; Fumagalli et al., 2009; Yadavilli et al., 2009; Zhang and Lu, 2009; Zhu et al., 2009; Daftuar et al., 2010; Xiong et al., 2011).

Here I suggest a novel extraribosomal function for RPS18A in the transition from vegetative to reproductive development. Concomitant with its role in transitional growth and development, *rps18a:HF/RPL18B* also effects bolt thickening, making this Arabidopsis r-protein double mutant unique among previously described mutants.

#### **CHAPTER 4. THE *rps18a:HF/RPL18B* DOUBLE MUTANT GENERATES A NOVEL PHENOTYPE – Relationship to the thesis in its entirety**

Chapter 4 relates to the thesis as it describes a non-typical r-protein double mutant, therefore, highlighting the complexity associated with r-proteins and their effects on plant growth and development.

## CHAPTER 5. GENERAL DISCUSSION AND CONCLUSIONS

The history of ribosome biology has revealed a number of substantial surprises. First, the demonstration that the large rRNA, not the r-protein, forms the catalytic core of the ribosome (Steitz et al., 1988). Second, the recognition of extraribosomal cellular functions of r-proteins distinct from the ribosome, such as roles in apoptosis, DNA repair, replication, transcription and as a regulator of the cell cycle (Wool, 1996; Takagi et al., 2005; Lindstrom, 2009). From the cross of a *rps18a* mutant to a *RPL18B* overexpressor a novel mutant phenotype was recorded. The late flowering/thickened bolt phenotype in the *rps18a:HF/RPL18B* double mutant was not produced in a similar *rps13a:HF/RPL18B* double mutant, suggesting a unique extraribosomal function specific to RPS18A. Adding more ammunition to the arsenal of surprises in plant ribosome research

Mutations in genes involved in ribosome biogenesis result in a strong phenotype, reminiscent of miRNA biogenesis and auxin synthesis and transport mutants; the *pointed first leaf* phenotype. Although the pathway to the *pfl* phenotype is far from understood, a model has been suggested to accommodate the similarities in mutant phenotypes exhibited by these three processes. The model suggests that efficient ribosome biogenesis leads to miRNA-mediated degradation of several auxin response genes culminating in the *pfl* phenotype (Degenhardt and Bonham-Smith, 2008).

Illumina sequencing of the transcriptome and miRNOME of *pfl1* (*rps18a*) and *pfl2* (*rps13a*) mutants has provided an unexpected layer of complexity to an already intricate ribosome biogenesis/plant development network. To confirm the proposed model, I expected transcriptome data to identify numerous genes involved in miRNA and auxin biogenesis pathways. I was surprised to find that most genes, up or down regulated, were involved in plant defense to multiple abiotic stresses, transposable elements, nitrogen metabolism or genes with as yet unknown function. Just as unexpected, miRNA profiling of the *pfl* mutants identified no differences in miRNA pools between the r-protein mutants and WT. Although preliminary, these data suggest a stronger link to genes involved in pathogen defense than to miRNA biogenesis and auxin homeostasis, once again highlighting the complex and unexpected nature of r-protein and ribosome biology in Arabidopsis.

Further complicating plant r-protein biology is the presence of multiple member r-protein gene families in which most members from each family are actively transcribed and incorporated into functional ribosomes (Barakat et al., 2001; Chang et al., 2005). The presence of multiple active isoforms provides the possibility of a massive level of ribosome heterogeneity; enabling plant ribosomes to fine-tune mRNA selection, as dictated by cellular environment and in response to developmental or environmental stresses. The only approach to dissecting the extent of ribosome heterogeneity is to determine the cellular requirement of each isoform from each r-protein gene family at any one time in the cell. While numerous individual r-protein mutants have been identified and studied (e.g. *rps18a*, *rps13a*, *rps27b*, *rpl24b* and *rpl28a*) (Byrne, 2009), little has been done with entire r-protein gene families (e.g. *RPL23a*, *RPL5* and *RPL4*) (Weijers et al., 2001; Degenhardt and Bonham-Smith, 2008; Rosado et al., 2010). To develop a complete representation for each r-protein family, mutational analysis and transcript/protein expression profiles under a variety of developmental stages and environmental conditions needs to be done. Until it is possible to assess r-protein content and transcript specificity of individual ribosomes, the aforementioned analyses will have to suffice.

Mutation analysis can readily be used for small Arabidopsis r-protein gene families, but complications arise when investigating larger families such as the *RPS15a* gene family. Containing six members, in two groups, type I (cytosolic) and type II (non-cytosolic), meaningful individual mutational analysis was difficult. While none of the individual r-protein KDs generated dramatic phenotype, except for reduced root length, both type I (*RPS15aA*, *D* and *F*) and type II (*RPS15aB* and *E*) family KDs were lethal. Using CSLM to visualize GFP-tagged RPS15aB and E, I confirmed that even though both are nuclear encoded r-proteins, they are not transported to the nucleolus for SSU assembly. Models suggesting that RPS15aB and E compensate for a loss of RPS8 in the mitochondria of angiosperms (Adams et al., 2002; Carroll et al., 2008) were not verified by CSLM. In fact, the weak cytoplasmic subcellular localization observed for both type II isoforms suggests that, like the acidic P r-proteins, RPS15aB and E are recruited to cytoplasmic ribosomes following SSU export from the nucleus. Complex families such as *RPS15a*, do not sit well within the ribosome heterogeneity model, as even though one of *RPS15aB* or *E* is absolutely

required for normal plant growth and development, we have no information to identify which ribosome population to which they belong.

Upon completion of this thesis, numerous questions remain. What are the roles of type II RPS15a isoforms? Which of the type I RPS15a isoforms are absolutely required in plant growth and development? Is there a common pathway affected between ribosome biogenesis, miRNA biogenesis and auxin synthesis and transport mutants? Does RPS18A possess an extraribosomal function in the cell? As was the case with this thesis, research addressing these questions is sure to reveal a great deal more about the complexity of r-protein biology.

## 6. APPENDIX A-LIST OF R-PROTEINS IDENTIFIED IN 15-DAY-OLD ARABIDOPSIS TOTAL PROTEIN

### 6.1. List of r-proteins identified in Arabidopsis seedlings by LC-MS/MS

**Table A.1: List of r-proteins identified in 15-day-old Arabidopsis total protein extract by LC-MS/MS at 15, 25 and 35 kDa.**

15 kDa Fragment	25 kDa Fragment	35 kDa Fragment
50S ribosomal protein L9, chloroplastic	60S ribosomal protein L7-2	60S ribosomal protein L7a-1
40S ribosomal protein S18	50S ribosomal protein L3-1, chloroplastic	40S ribosomal protein S6-2
50S ribosomal protein L11, chloroplastic	60S ribosomal protein L7-3	40S ribosomal protein S6-1
40S ribosomal protein S13-2	50S ribosomal protein L5, chloroplastic	60S ribosomal protein L8-1
40S ribosomal protein S13-1	60S ribosomal protein L10-1	50S ribosomal protein L4, chloroplastic
50S ribosomal protein L12-1	50S ribosomal protein L15, chloroplastic	40S ribosomal protein S3a-1
40S ribosomal protein S14-2	60S ribosomal protein L10a-2	40S ribosomal protein S3-1
40S ribosomal protein S19-1	60S ribosomal protein L10a-1	60S ribosomal protein L7a-2
60S ribosomal protein L27a-3	60S ribosomal protein L10-3	50S ribosomal protein L2, chloroplastic
40S ribosomal protein S16-3	Ribosome-recycling factor, chloroplastic	60S ribosomal protein L8-3
60S ribosomal protein L27a-2	60S ribosomal protein L13-1	40S ribosomal protein S2-3
40S ribosomal protein S16-1	30S ribosomal protein S3, chloroplastic	40S ribosomal protein S4-1
60S ribosomal protein L23	60S ribosomal protein L10a-3	40S ribosomal protein S3-2
50S ribosomal protein L29, chloroplastic	30S ribosomal protein S2, chloroplastic	50S ribosomal protein L1, chloroplastic
60S ribosomal protein L28-1	60S ribosomal protein L15-2	40S ribosomal protein S4-3
30S ribosomal protein 3-1	60S ribosomal protein L15-1	60S ribosomal protein L6-3
40S ribosomal protein S15-4	60S ribosomal protein L19-1	40S ribosomal protein S3a-2
40S ribosomal protein S15-1	60S ribosomal protein L13-3	40S ribosomal protein S3-3
40S ribosomal protein S19-3	60S ribosomal protein L19-3	40S ribosomal protein S8-1
40S ribosomal protein S19-2	60S ribosomal protein L19-2	40S ribosomal protein S2-1
60S acidic ribosomal protein P2-1	60S ribosomal protein L6-3	60S ribosomal protein L6-1
40S ribosomal protein S24-1	60S acidic ribosomal protein P0-2	30S ribosomal protein S5, chloroplastic
40S ribosomal protein S23-2	60S ribosomal protein L6-1	60S ribosomal protein L8-2
40S ribosomal protein S17-2	Ribosomal protein S2, mitochondrial	40S ribosomal protein S2-4

30S ribosomal protein S7, chloroplastic  
60S ribosomal protein L32-2  
60S ribosomal protein L14-2  
60S ribosomal protein L32-1  
40S ribosomal protein S24-2  
50S ribosomal protein L27, chloroplastic  
60S acidic ribosomal protein P2-2  
50S ribosomal protein L24, chloroplastic  
60S ribosomal protein L35-1  
30S ribosomal protein S9, chloroplastic  
40S ribosomal protein S23-1  
60S ribosomal protein L36-3  
40S ribosomal protein S12-1  
40S ribosomal protein S17-1  
40S ribosomal protein S26-3  
60S ribosomal protein L36-2  
40S ribosomal protein S10-3  
30S ribosomal protein S11, chloroplastic  
30S ribosomal protein S17, chloroplastic  
50S ribosomal protein L16, chloroplastic  
40S ribosomal protein S20-1  
40S ribosomal protein S26-2  
60S ribosomal protein L35-2  
40S ribosomal protein S10-1  
60S ribosomal protein L35-4  
40S ribosomal protein S20-2  
40S ribosomal protein S26-1  
60S ribosomal protein L27-3  
60S ribosomal protein L35-3  
50S ribosomal protein L22, chloroplastic  
60S ribosomal protein L23a-1

60S ribosomal protein L8-1  
40S ribosomal protein S3a-2  
Putative 60S ribosomal protein L18-1

40S ribosomal protein S8-2  
60S ribosomal protein L19-1  
40S ribosomal protein Sa-1  
60S acidic ribosomal protein P0-1



60S ribosomal protein L34-3  
60S ribosomal protein L22-2  
60S ribosomal protein L26-1  
60S ribosomal protein L34-1  
60S acidic ribosomal protein P1-1  
60S acidic ribosomal protein P3-2  
60S ribosomal protein L36-1  
60S ribosomal protein L34-2  
60S ribosomal protein L35a-2  
50S ribosomal protein L17  
60S ribosomal protein L28-2  
60S acidic ribosomal protein P1-3  
60S ribosomal protein L31-3  
40S ribosomal protein S3-3  
40S ribosomal protein S11-1  
40S ribosomal protein S11-3  
50S ribosomal protein 5, chloroplastic  
60S ribosomal protein L24-1  
60S ribosomal protein L22-3  
60S ribosomal protein L31-1  
40S ribosomal protein S12-2  
40S ribosomal protein S10-2  
60S ribosomal protein L36a  
30S ribosomal protein S8, chloroplastic  
30S ribosomal protein 3-2, chloroplastic  
60S acidic ribosomal protein P1-2  
Putative 60S ribosomal protein L22-1  
60S ribosomal protein L8-3  
30S ribosomal protein S12, chloroplastic  
60S ribosomal protein L37-3

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## 7. APPENDIX B- LIST OF ILLUMINA IDENTIFIED UP AND DOWN REGULATED TRANSCRIPTS UNIQUE TO *pfl1* AND *pfl2*

### 7.1. *pfl1* specific up and/or down regulated genes identified by Illumina sequencing

**Table B.1: Transcripts up or down regulated unique to *pfl1* as identified by Illumina sequencing.**

<i>pfl1</i> Unique Genes	
Gene Locus and Function	Fold Change
>AT2G14610.1   Symbols: PR1, PR 1, ATRP1   pathogenesis-related gene 1	9.793494
>AT1G54040.1   Symbols: ESP, TASTY, ESR   epithiospecifier protein	9.556339
>AT4G29200.1   Symbols:   Beta-galactosidase related protein	Turned On
>AT1G54040.2   Symbols: ESP, TASTY   epithiospecifier protein	8.993842
>AT1G75940.1   Symbols: ATA27, BGLU20   Glycosyl hydrolase superfamily protein	8.909591
>AT2G05520.3   Symbols: GRP-3, ATGRP-3   glycine-rich protein 3	7.746158
>AT5G24780.2   Symbols: VSP1, ATVSP1   vegetative storage protein 1	8.208531
>AT2G05520.2   Symbols: GRP-3, ATGRP-3   glycine-rich protein 3	7.369882
>AT2G13665.2   Symbols:   other RNA	Turned On
>AT3G61111.1   Symbols:   Zinc-binding ribosomal protein family protein	Turned On
>AT5G24735.1   Symbols:   other RNA	0
>AT2G39030.1   Symbols:   Acyl-CoA N-acyltransferases (NAT) superfamily protein	Turned On
>AT5G23840.1   Symbols:   MD-2-related lipid recognition domain-containing protein	Turned On
>AT1G24996.1   Symbols:   unknown protein	0
>AT2G21330.1   Symbols: FBA1   fructose-bisphosphate aldolase 1	6.885504
>AT5G09660.1   Symbols: PMDH2   peroxisomal NAD-malate dehydrogenase 2	6.784811
>AT3G50460.1   Symbols: HR2   homolog of RPW8 2	Turned On
>AT1G72930.2   Symbols: TIR   toll/interleukin-1 receptor-like	6.779998
>AT5G40370.2   Symbols:   Glutaredoxin family protein	6.694342
>AT2G05642.1   Symbols:   Nucleic acid-binding, OB-fold-like protein	Turned On
>AT4G08870.1   Symbols:   Arginase/deacetylase superfamily protein	0
>AT3G48640.1   Symbols:   unknown protein	6.769109
>AT4G04402.1   Symbols:   two-component phosphorelay mediator, putative	Turned On

>AT1G43170.9   Symbols: RP1   ribosomal protein 1	6.678912
>AT1G43170.7   Symbols: RP1   ribosomal protein 1	6.406539
>AT2G05520.6   Symbols: GRP-3, ATGRP-3   glycine-rich protein 3	6.117033
>AT1G16410.2   Symbols: CYP79F1, BUS1, SPS1   cytochrome p450 79f1	6.128438
>AT1G43170.6   Symbols: RP1   ribosomal protein 1	6.349206
>AT2G21330.3   Symbols: FBA1   fructose-bisphosphate aldolase 1	6.069899
>AT5G23840.2   Symbols:   MD-2-related lipid recognition domain-containing protein	Turned On
>AT5G25980.1   Symbols: TGG2, BGLU37   glucoside glucohydrolase 2	5.961755
>AT5G53700.1   Symbols:   RNA-binding (RRM/RBD/RNP motifs) family protein	Turned On
>AT1G14880.1   Symbols: PCR1   PLANT CADMIUM RESISTANCE 1	6.024839
>AT2G14560.1   Symbols: LURP1   Protein of unknown function	5.939728
>AT1G24390.1   Symbols:   unknown protein	Turned On
>AT1G02930.1   Symbols: ATGSTF6, GST1, ERD11, ATGSTF3, GSTF6, ATGST1   glutathione S-transferase 6	6.425155
>AT4G25100.3   Symbols: FSD1   Fe superoxide dismutase 1	5.88122
>AT2G18660.1   Symbols: PNP-A   plant natriuretic peptide A	5.96503
>AT1G43170.5   Symbols: RP1   ribosomal protein 1	6.099755
>AT1G65990.1   Symbols:   type 2 peroxiredoxin-related / thiol specific antioxidant / mal allergen family protein	Turned On
>AT1G09486.1   Symbols:   pseudogene, 60S ribosomal protein L21 (RPL21B)	5.711505
>AT4G22710.1   Symbols: CYP706A2   cytochrome P450, family 706, subfamily A, polypeptide 2	5.853998
>AT5G10140.1   Symbols: FLC, FLF, AGL25   K-box region and MADS-box transcription factor family protein	6.89464
>AT3G22235.2   Symbols:   FUNCTIONS IN: molecular_function unknown	5.76285
>AT3G53740.3   Symbols:   Ribosomal protein L36e family protein	6.15098
>AT3G52300.2   Symbols: ATPQ   ATP synthase D chain, mitochondrial	5.678912
>AT2G05520.4   Symbols: GRP-3, ATGRP-3   glycine-rich protein 3	5.605611

## 7.2. *pfl2* specific up and/or down regulated genes identified by Illumina sequencing

**Table B.2: Transcripts up or down regulated unique to *pfl2* as identified by Illumina sequencing.**

<i>pfl2</i> Unique Genes	
Gene Locus and Function	Fold Change
>AT1G67105.1   Symbols:   other RNA	8.630129
>AT4G32230.1   Symbols:   unknown protein	Turned On
>AT2G05914.1   Symbols:   Potential natural antisense gene	8.236066
>AT1G64770.3   Symbols: NDF2   NDH-dependent cyclic electron flow 1	7.712711
>AT3G08940.1   Symbols: LHCB4.2   light harvesting complex photosystem II	6.608301
>AT5G47700.2   Symbols:   60S acidic ribosomal protein family	6.521858
>AT1G31580.1   Symbols: ECS1, CXC750   ECS1	-6.672854
>AT2G06950.1   Symbols:   transposable element gene	0
>AT3G44042.1   Symbols: SADHU3-1   transposable element gene	Turned On
>AT1G58270.1   Symbols: ZW9   TRAF-like family protein	0
>AT4G13940.1   Symbols: HOG1, EMB1395, SAHH1, MEE58, ATSAHH1   S-adenosyl-L-homocysteine hydrolase	6.084247
>AT3G06700.3   Symbols:   Ribosomal L29e protein family	6.10578
>AT3G15353.2   Symbols: MT3   metallothionein 3	5.969064
>AT4G03050.1   Symbols: AOP3   2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	Turned On
>AT3G62475.1   Symbols:   transposable element gene	7.263953
>AT3G09200.2   Symbols:   Ribosomal protein L10 family protein	6.035884
>AT5G01750.1   Symbols:   Protein of unknown function	6.250897
>AT4G20260.3   Symbols: ATPCAP1, PCAP1   plasma-membrane associated cation-binding protein 1	6.497443
>AT4G22890.1   Symbols: PGR5-LIKE A   PGR5-LIKE A	6.033305
>AT1G64940.1   Symbols: CYP89A6   cytochrome P450, family 87, subfamily A, polypeptide 6	6.646126
>AT4G16830.1   Symbols:   Hyaluronan / mRNA binding family	6.632767
>AT5G15960.1   Symbols: KIN1   stress-responsive protein (KIN1) / stress-induced protein (KIN1)	6.28716
>AT4G00413.1   Symbols:   pseudogene, similar to tomato ripening associated membrane protein	Turned On
>AT4G17585.1   Symbols:   Aluminium activated malate transporter family protein	Turned On

>AT5G60600.3	Symbols: GCPE, ISPG, CSB3, CLB4, HDS   4-hydroxy-3-methylbut-2-enyl diphosphate synthase	5.854968
>AT1G44575.2	Symbols: NPQ4, PSBS   Chlorophyll A-B binding family protein	5.436969
>AT1G20620.2	Symbols: CAT3, SEN2, ATCAT3   catalase 3	5.711123
>AT1G23130.1	Symbols:   Polyketide cyclase/dehydrase and lipid transport superfamily protein	-5.804259
>AT1G62180.2	Symbols: APR2   5'adenylylphosphosulfate reductase 2	5.603661
>AT3G53460.1	Symbols: CP29   chloroplast RNA-binding protein 29	5.584198
>AT4G20480.1	Symbols:   Putative endonuclease or glycosyl hydrolase	0
>AT3G54050.2	Symbols: HCEF1   high cyclic electron flow 1	5.371444
>AT2G28550.1	Symbols: RAP2.7, TOE1   related to AP2.7	6.048476
>AT1G61520.3	Symbols: LHCA3   photosystem I light harvesting complex gene 3	5.488396
>AT5G38660.2	Symbols: APE1   acclimation of photosynthesis to environment	6.211004
>AT4G20260.2	Symbols: ATPCAP1, PCAP1   plasma-membrane associated cation-binding protein 1	6.163024
>AT3G30720.1	Symbols: QQS   qua-quine starch	0
>AT1G33090.1	Symbols:   MATE efflux family protein	Turned On
>AT2G05915.1	Symbols:   unknown protein	6.659362
>AT2G05380.2	Symbols: GRP3S   glycine-rich protein 3 short isoform	5.44529
>AT5G01810.1	Symbols: CIPK15, ATPK10, PKS3, SNRK3.1, SIP2   CBL-interacting protein kinase 15	5.561237
>AT4G22890.4	Symbols: PGR5-LIKE A   PGR5-LIKE A	5.541487
>AT4G14030.2	Symbols: SBP1   selenium-binding protein 1	5.537582
>AT5G02870.2	Symbols:   Ribosomal protein L4/L1 family	5.299894
>AT3G47965.1	Symbols:   unknown protein	0
>AT5G03090.1	Symbols:   BEST Arabidopsis thaliana protein match is: mto 1 responding down 1	5.875529
>AT1G03106.1	Symbols:   unknown protein	Turned On
>AT5G41700.4	Symbols: UBC8, ATUBC8   ubiquitin conjugating enzyme 8	6.169977
>AT5G60600.1	Symbols: GCPE, ISPG, CSB3, CLB4, HDS	5.557
>AT1G15400.1	Symbols:   unknown protein	6.160699
>AT5G55570.1	Symbols:   unknown protein; LOCATED IN: chloroplast	5.085718
>AT5G02270.2	Symbols: NAP9   non-intrinsic ABC protein 9	5.626042
>AT4G20440.1	Symbols: smB   small nuclear ribonucleoprotein associated protein B	5.434038
>AT4G07825.1	Symbols:   unknown protein	0
>AT4G03520.2	Symbols: ATHM2   Thioredoxin superfamily protein	5.17649

>AT5G28919.1	Symbols:   FUNCTIONS IN: molecular_function unknown	0
>AT1G67700.3	Symbols:   unknown protein; FUNCTIONS IN: molecular_function unknown	5.048763
>AT4G22890.5	Symbols: PGR5-LIKE A   PGR5-LIKE A	5.302427
>AT2G23130.2	Symbols: AGP17, ATAGP17   arabinogalactan protein 17	5.982607
>AT2G21580.2	Symbols:   Ribosomal protein S25 family protein	5.145998
>AT5G04380.1	Symbols:   S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	6.364355
>AT5G66040.2	Symbols: STR16   sulfurtransferase protein 16	4.954504
>AT4G22890.2	Symbols: PGR5-LIKE A   PGR5-LIKE A	5.818265
>AT4G20260.6	Symbols: PCAP1   plasma-membrane associated cation-binding protein 1	5.818265
>AT1G70830.1	Symbols: MLP28   MLP-like protein 28	6.827089
>AT4G19240.1	Symbols:   unknown protein	Turned On
>AT5G17170.2	Symbols: ENH1   rubredoxin family protein	5.144137
>AT4G03050.2	Symbols: AOP3   2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	Turned On
>AT3G16410.1	Symbols: NSP4   nitrile specifier protein 4	5.197458
>AT4G38810.1	Symbols:   Calcium-binding EF-hand family protein	5.423733
>AT1G05530.1	Symbols: UGT75B2, UGT2   UDP-glucosyl transferase 75B2	6.289716
>AT5G25980.2	Symbols: TGG2, BGLU37   glucoside glucohydrolase 2	-5.909841
>AT3G17800.2	Symbols:   Protein of unknown function	5.164924
>AT5G45775.1	Symbols:   Ribosomal L5P family protein	5.08651
>AT3G53420.2	Symbols: PIP2A, PIP2, PIP2;1	4.67303
>AT4G20260.1	Symbols: ATPCAP1, PCAP1	5.306038
>AT5G13630.2	Symbols: GUN5, CCH, CHLH, CCH1, ABAR	4.740481
>AT1G52870.1	Symbols:   Peroxisomal membrane 22 kDa (Mpv17/PMP22) family protein	5.669209
>AT1G27385.1	Symbols:   unknown protein	5.084247
>AT5G28920.1	Symbols:   unknown protein	0
>AT3G44110.2	Symbols: ATJ3, ATJ   DNAJ homologue 3	5.157593
>AT1G04270.2	Symbols: RPS15   cytosolic ribosomal protein S15	5.078346
>AT4G13940.3	Symbols: HOG1, SAHH1   S-adenosyl-L-homocysteine hydrolase	4.774048
>AT5G60600.2	Symbols: GCPE, ISPG, CSB3, CLB4, HDS   4-hydroxy-3-methylbut-2-enyl diphosphate synthase	5.156038
>AT1G35500.1	Symbols:   unknown protein	Turned On
>AT2G22670.1	Symbols: IAA8   indoleacetic acid-induced protein 8	5.742556

>AT3G44590.2   Symbols:   60S acidic ribosomal protein family	5.742556
>AT5G66052.1   Symbols:   unknown protein	0
>AT1G01620.2   Symbols: PIP1C, TMP-B, PIP1;3   plasma membrane intrinsic protein 1C	4.781123
>AT1G68010.2   Symbols: HPR   hydroxypyruvate reductase	4.698356
>AT3G21380.1   Symbols:   Mannose-binding lectin superfamily protein	Turned On
>AT1G67700.1   Symbols:   unknown protein	4.667074
>AT1G20020.3   Symbols: ATLFNR2   ferredoxin-NADP(+)-oxidoreductase 2	4.934918
>AT3G44430.1   Symbols:   unknown protein	0
>AT1G53480.1   Symbols: ATMRD1, MRD1   mto 1 responding down 1	6.513931
>AT1G66260.1   Symbols:   RNA-binding (RRM/RBD/RNP motifs) family protein	5.513931
>AT5G06280.3   Symbols:   unknown protein	5.160699
>AT5G48860.1   Symbols:   unknown protein	Turned On

## 8. APPENDIX C. LIST OF OLIGONUCLEOTIDE PRIMERS

**Table C.1: List of oligonucleotide primers used for RNAi constructs**

Gene	Primer	Primer Sequence	Restriction Site
RPS15aA sense	FORWARD	GCGCTCGAGCTATTGAAGTTTTATTTTATGGG	XhoI
	REVERSE	GCGGGTACCTCCGAAGTCTAAAAAAGGGCTCC	KpnI
RPS15aA anti	FORWARD	GCGTCTAGACTATTGAAGTTTTATTTTATGG	XbaI
	REVERSE	GCGAAGCTTTCCGAAGTCTAAAAAAGGGCTCC	HindIII
RPS15aD sense	FORWARD	GCGCTCGAGATCTATCTTATCGGGTTATAGTT	XhoI
	REVERSE	GCGGGTACCAAGATGATTTTGATTTCATAAAT	KpnI
RPS15aD anti	FORWARD	GCGTCTAGAATCTATCTTATCGGGTTATAGTT	XbaI
	REVERSE	GCGAAGCTTAAGATGATTTTGATTTCATAAAT	HindIII
RPS15aF sense	FORWARD	GCG CTCGAG TCAAGTCTTGAAGAAGAGAATCA	XhoI
	REVERSE	GCG GGTACC CTACTTTGCAAGTCTTTAACAAC	KpnI
RPS15aF anti	FORWARD	GCG GGATCC TCAAGTCTTGAAGAAGAGAATCA	BamHI
	REVERSE	GCG ATCGAT CTACTTTGCAAGTCTTTAACAAC	Clal
RPS15aB sense	FORWARD	GCGCTCGAGTTCTTTCATTGAACTGCAATACG	XhoI
	REVERSE	GCGGGTACCAGTGCAAGAAAACAAACAAACAA	KpnI
RPS15aB anti	FORWARD	GCGTCTAGATTCTTTCATTGAACTGCAATACG	XbaI
	REVERSE	GCGAAGCTTAGTGCAAGAAAACAAACAAACAA	HindIII
RPS15aE sense	FORWARD	GCGCTCGAGCTTGAGCTAGCACATGGCAAGAA	XhoI
	REVERSE	GCGGGTACCAAATTTCTCGTCCAAAATATAAA	KpnI
RPS15aE anti	FORWARD	GCGTCTAGACTTGAGCTAGCACATGGCAAGAA	XbaI
	REVERSE	GCGAAGCTTAAATTTCTCGTCCAAAATATAAA	HindIII
RPS15aA/D/F sense	FORWARD	GCG CTCGAG ATGGTAAGAATCAGTGTTCTT	XhoI
	REVERSE	GCG GGTACC AAGAAGCCGAGAACCTTGCCT	KpnI
RPS15aA/D/F anti	FORWARD	GCG GGATCC ATGGTAAGAATCAGTGTTCTT	BamHI
	REVERSE	GCG ATCGAT AAGAAGCCGAGAACCTTGCCT	Clal



RPS15aB/E sense	FORWARD	GCG CTCGAG AAA CCA ATC TCC ACC GTT ATG TC	XhoI
	REVERSE	GCG GGTACC CCA AGA ACC TGA CCA CCC ACA	KpnI
RPS15aB/E anti	FORWARD	GCG TCTAGA AAA CCA ATC TCC ACC GTT ATG TC	XbaI
	REVERSE	GCG GGATCC CCA AGA ACC TGA CCA CCC ACA	BamHI

#### Hairpin Sequencing Primers

P-5 sense	FORWARD	GGGATGACGCACAATCC
P-5 sense	REVERSE	GAGCTACACATGCTCAGG
I-5 anti	FORWARD	ATAATCATACTAATTAACATCAC
I-3 anti	REVERSE	TGATAGATCATGTCATTGTG

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**Table C.2: List of oligonucleotide primers used for subcellular localization experiments**

Gene	Primer	Primer Sequence	Restriction Site
pBINRPS15aB	FORWARD	GCG GGATCC ATGGGGAGGAGAATATTG	BamHI
	REVERSE	GCG ACTAGT ATGAAAGAAACCAAG	SpeI
pBINRPS15aE	FORWARD	GCG GGATCC ATGGGGAGGAGGATTTTG	BamHI
	REVERSE	GCG ACTAGT GTAAAAGAAGCCAAGA	SpeI
pGREENRPS15aA/F	FORWARD	GCGGAATTCATGGTAAGAATCAGTGTTT	EcoRI
	REVERSE	GCGGGATCCATAGAAGAAGCCGAGAACC	BamHI
pGREENRPS15aD	FORWARD	GCGGAATTCATGGTGAGAATCAGTGTC	EcoRI
	REVERSE	GCGGGATTCGTAAAAGAACCCAAGAAC	BamHI
pGREENRPS15aB	FORWARD	GCGGAATTCATGGGGAGGAGAATATTG	EcoRI
	REVERSE	GCGGGATCCATGAAAGAAACCAAG	BamHI
pGREENRPS15aE	FORWARD	GCGGAATTCATGGGGAGGAGGATTTTG	EcoRI
	REVERSE	GCGGGATCCGTAAAAGAAGCCAAGA	BamHI
pGREENRPS18A	FORWARD	GCGGAATTC ATGTCTCTAG TTGCGAACG	EcoRI
	REVERSE	GCGGGATCC ACGCTTCTTG GAAACACC	BamHI
pGREENRPS18B	FORWARD	GCGGAATTC ATGTCTCTGG TTGCAAATG	EcoRI
	REVERSE	GCGGGATCC ACGCTTCTTA GACACACC	BamHI
pGREENRPS18C	FORWARD	GCGGAATTC ATGTCTCTGG TTGCAAATG	EcoRI
	REVERSE	GCGGGATCC ACGCTTCTTT GAGACACC	BamHI
pGREENRPS13A	FORWARD	GCGCAATTG ATGGGTGCGA TGCATAGT	MfeI
	REVERSE	GCGGGATCC AGCCACAAGA GTGCTTGC	BamHI
pGREENRPS13B	FORWARD	GCGGAATTC ATGGGTGCGA TGCACAGTCG	EcoRI
	REVERSE	GCGGGATCC AGCCACAAGG GTGCTCG	BamHI
pGREENRPL18B	FORWARD	GCGCAATTG ATGGGTATTG ATCTGATCG	MfeI
	REVERSE	GCGGGATCC AACCTTGAAT CCACG	BamHI
pGREENRPL18C	FORWARD	GCGGAATTC ATGGGTATCG ATCTTATCG	EcoRI
	REVERSE	GCGGGATCC GACCTTGAAT CCACGAC	BamHI

**Table C.3: List of oligonucleotide primers used in RPS15aD/F C-terminal deletion constructs**

Gene	Primer	Primer Sequence	Restriction Site
S15aDF	FORWARD	GCGGAATTCATGGTGAGAATCAGTGTGCTC	BamHI
S15aDR	REVERSE	GCGGGATCCGTAAAAGAACCCAAGAAC	EcoRI
S15aDΔ1R	REVERSE	GCGGAATCCACCAAAGTGTCTGGAAGG	EcoRI
S15aDΔ2R	REVERSE	GCGGGATCCGGTCGTCAGAACAAATGAA	EcoRI
S15aDΔ3R	REVERSE	GCGGAATCCTCCCGCTGAGGTCGTCAG	EcoRI
S15aDΔ4R	REVERSE	GCGGGATCCGCCACCAACGTTCTGTTCT	EcoRI
S15aFF	FORWARD	GCGGAATTCATGGTGAGAATCAGTGTGCTTAA	BamHI
S15aFR	REVERSE	GCGGGATCCTAAAAGAAGCCAAGAAC	EcoRI
S15aFΔ1R	REVERSE	GCGGGATCCCCCAAAGTGTCTGGAAGG	EcoRI
S15aFΔ2R	REVERSE	GCGGGATCCGGTCGTAAGAACAATGTA	EcoRI
S15aFΔ3R	REVERSE	GCGGGATCCACCAGCCGAGGTCGTAAG	EcoRI
S15aFΔ4R	REVERSE	GCGGGATCCGCCACCAACATTCTTTCT	EcoRI

**Table C.4: List of oligonucleotide primers used in Chapter 2 qRT-PCR experiments**

Gene	Primer	Primer Sequence
<i>RPS15aA</i>	FORWARD	GCGGGAAACAGAT TTTCATTGAG
	REVERSE	GCGTCCGAAGTCTAAAAAAGGGC
<i>RPS15aD</i>	FORWARD	GCGTCGAGATCTATCTTATCGGG
	REVERSE	GCGCTAACGAATTCAAAAAGTGA
<i>RPS15aF</i>	FORWARD	GCGAAATGTTTAAGGGTAGTAGA
	REVERSE	GCGTTTTGTTGTAGAAAACAGAA
<i>RPS15aB</i>	FORWARD	GCGGCAATACGGAAAGAACTCT
	REVERSE	GCGATTTTGTTCTCTAATTCTCC
<i>RPS15aE</i>	FORWARD	GCGCATGGCAAGAACCTTCTAGT
	REVERSE	GCGAGATCTTTGAATTTAGCAAC
<i>ACTIN7</i>	FORWARD	GATATTCAGCCACTTGTCTGTGAC
	REVERSE	CATGTTCGATTGGATACTTCAGAG

**Table C.5: List of oligonucleotide primers used in Chapter 3 qRT-PCR experiments**

Gene	Primer	Primer Sequence
<i>B-GLUCOSIDASE18-1</i>	FORWARD	GCGTAATTGAGTACAAGAGTCT
	REVERSE	GCGTGGTGGCTGAGCTCTATA
<i>B-GLUCOSIDASE18-2</i>	FORWARD	GCGTAATTGAGTACAAGAGACT
	REVERSE	GCGCGAACCAGTTGAGTGAAGG
<i>PYK10-BINDING PROTEIN</i>	FORWARD	GCGCCATGTTTCGTCCTTTATCCAACTG
	REVERSE	GCGACTTCCTTTATTAAAATAGAGCAC
<i>TIR1</i>	FORWARD	GCGATCAAAAATC AAAATGTCTT CTC
	REVERSE	GCGAGCTCTTTGTCGTCTTTGAAGGTTCTG
<i>RIBOSOMAL PROTEIN1</i>	FORWARD	GCGAGTCCTCATCGTTGGAGCTT
	REVERSE	GCGACGATGTGAGTCATACCAGC
<i>TRANSPOSABLE ELEMENT1</i>	FORWARD	GCGACAACGGGCAAAGATGGAGT
	REVERSE	GCGTTCAAACAGTTTCAGGGTTTCAAACAGG
<i>SADHU1-1</i>	FORWARD	GCGGCTCAAGCCAAAAGTTTGATGTCGAGGTCTG
	REVERSE	GCGAATCCCAAATGGGCTTATGGAAGATGGGCC
<i>GLUTAMINE SYNTHETASE2A</i>	FORWARD	AACCGTGGATGCTCTATTCGTGT
	REVERSE	AGAAAGCTTTTGAGCTGCAAGGGCTT
<i>GLUTAMINE SYNTHETASE2B</i>	FORWARD	GCGATTTCGGATTAAGACATTGTTTGTTGTC
	REVERSE	GCGAAGCTTATCATTTAATTTAAATGAAAGCCC
<i>OXIDOREDUCTASE</i>	FORWARD	GCGCTCTTCTTAAACCTCATCC ACCC
	REVERSE	GCGACAGCAGCATTTCATGGTAGTCGCC
<i>UNKNOWN PROTEIN1</i>	FORWARD	GCGGCCTATTCCGGAATGTCTTCG
	REVERSE	GCGGAAGCCACAAACCAAACCACAATG
<i>UNKNOWN PROTEIN2</i>	FORWARD	GAAGAGCAAGCCAGATTTGAACACCA
	REVERSE	TACACTCAAGTATTTAAAGAAGATCA
<i>UNKNOWN PROTEIN4</i>	FORWARD	GCGGCCTATTCCGGAATGTCTTCG
	REVERSE	GCGGGAAGCCACAAACCAAACCACAATG
<i>LURP1</i>	FORWARD	GCGTGAGGTGTTTTGGCTAACAACGTAGAGG
	REVERSE	GCGCTTTATTTCGATATTTAGAAAATATATG
<i>ACTIN7</i>	FORWARD	GATATTCAGCCACTTGTCTGTGAC

REVERSE

CATGTTGATTGGATACTTCAGAG

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